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Light and Water Induced Morphological Changes in Arabidopsis thaliana (L.) Heynh. Plasticity and Selection

Anna Maria Kolodynska
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To the Graduate Council:

I am submitting herewith a dissertation written by Anna Maria Kolodynska entitled "Light and Water Induced Morphological Changes in Arabidopsis thaliana (L.) Heynh. Plasticity and Selection." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.

Massimo Pigliucci, Major Professor

We have read this dissertation and recommend its acceptance:

Otto J. Schwarz, Mitchell Cruzan, Randall Small

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

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Otto J. Schwarz

Mitchell Cruzan

Randall Small

Accepted for the Council:

Anne Mayhew
Vice Provost and Dean of Graduate Studies

(Original Signatures are on file in the Graduate Student Servies Office)

LIGHT AND WATER INDUCED MORPHOLOGICAL CHANGES IN *ARABIDOPSIS THALIANA* (L.) HEYNH. PLASTICITY AND SELECTION.

A Dissertation
Presented for the
Degree of Doctor of Philosophy
The University of Tennessee, Knoxville

Anna Maria Kolodynska
May 2002

DEDICATION

To all who I love

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ABSTRACT

Phenotypic plasticity, the effect of plastic traits on fitness, and the stability of genetic variance-covariance matrices were investigated in an artificial population of *Arabidopsis thaliana* in response to environmental stress. These questions were also investigated in a selection experiment, where plants were subjected to selection on reproductive fitness (fruit production) under flooded and non-flooded water regimes.

Forty-seven accessions of early flowering *A. thaliana* were grown in laboratory conditions and were subjected to environmental stress such as low and medium light intensity, flooded and non-flooded water regimes, and a factorial arrangement of these light and water stresses. In addition, soft selection was applied for three generations in order to investigate *patterns of* phenotypic plasticity and phenotypic integration *expressed in response to these stressful environments*. Data was analyzed using a mixed model for the analysis of variance, linear regression was used for calculating the effects of plastic traits on reproductive fitness, and principal component analysis and vector correlations were used to investigate the stability of genetic variance-covariance matrices.

Results indicated that there was little plasticity to the environmental stresses applied, but there was a high degree of genetic variation to these environmental factors among accessions. Plastic traits had varying effects on reproductive fitness, in general there was selection for the increase of trait values, except for the bolting time which was under negative selection. When environmental stresses were manipulated independently, genetic variance-covariance matrices tended to stable. In contrast, when the environmental stresses were manipulated in a factorial arrangement, these matrices were less stable.

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PART I:

Phenotypic plasticity to light intensity in *Arabidopsis thaliana*: invariance of reaction norms and phenotypic integration

Statement:

This part of the dissertation was submitted for publication to the Journal of Evolutionary Biology. All tables and figures are located in the Appendix I.

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Introduction

Phenotypic plasticity is the property of a genotype to produce different phenotypes in response to distinct environments (Schmalhausen 1949). As sessile organisms, plants experience unavoidable fluctuations in the conditions of the external environment, a situation that can lead to

the evolution of phenotypic plasticity in a variety of characters (Bradshaw 1972). Furthermore, different environments are known to induce distinct patterns of character correlations (Schlichting and Levin 1986), although it is not clear to what extent this is a result of natural selection for "phenotypic integration" (Wagner 1995) or of inevitable genetic constraints in the form of pleiotropy or linkage (Cheverud 1984).

Light is a fundamental heterogeneous environmental factor for plants, and different aspects of light availability (quantity, daylength, spectral quality, angle of incidence) are perceived by specialized photoreceptors (Ballare` 1999; Lasceve et al. 1999) that induce responses that are considered adaptive (Schmitt et al. 1999). For example, plasticity in leaf morphology induced by light quantity is one of the best-known responses to environmental heterogeneity (e.g., Nunez-Olivera et al. 1996).

In this paper, we focus on genetic differentiation among populations for plasticity to light quantity and on light-induced changes in patterns of character correlations (phenotypic integration) in *Arabidopsis thaliana* (Brassicaceae). This plant is now an established model system in molecular biology and physiology (Anderson and Roberts 1998; Kunkel 1996), and has recently received much attention from an ecological and evolutionary standpoint (reviewed in Pigliucci 1998). Numerous studies have been published on light-induced responses in *A. thaliana* at the molecular or physiological levels (e.g., Lasceve et al. 1999; Whitelam and Devlin 1998). However, far fewer papers have focused on organismal studies and on ecologically relevant conditions (e.g., Nooden et al. 1996; Petrov and Petrosov 1981). Furthermore, the great majority of studies of light responses in *A. thaliana*, at both the molecular and organismal levels, have thus far dealt with spectral quality (Whitelam et al. 1998), at the detriment of our understanding of the mechanisms and significance of plasticity to other components of the light environment. This is important because distinct aspects of light availability are perceived differently by plants, so that

separating these components under controlled conditions is not just a convenient experimental device.

We present a study on the reaction norms to light quantity in a large collection of wild type populations of *A. thaliana*. We concentrate on both genetic differentiation of reaction norms of a variety of individual traits and on environment-induced changes in the patterns and degrees of phenotypic integration of the same traits. It is important to realize that biological parameters such as means and correlations measured across populations (as in this case) have a different meaning from their within-population equivalents (Armbruster and Schwaegerle 1996). Within-population genetic architecture is a good predictor of the immediate response of that population to selection, although it also is at the same time the result of previous short-term selective history, drift and migration. Across-population genetic architectures, on the other hand, tell us about the medium term *outcome* of past selective and historical forces that have sorted combinations of genotypes (and their pleiotropic and epistatic effects) in different populations across the species range. This is because selection acts within populations (save for the possibility of group selection, not considered here) and across-population genetic architecture is a compound measure of within-population genetic parameters. Since across-population genetic correlations are conceptually analogous to inter-specific allometry (Riska 1991; Klingenberg and Zimmermann 1992) they reflect the divergence among populations rather than the potential for response to selection. It is the environmental lability of this latter type of correlation structure that we wish to investigate here. This intermediate level of analysis is important as it provides a link between the micro-evolutionary phenomena that are the focus of population biology and the macro-evolutionary patterns observable at and above the species level (Hansen and Martins 1996).

When addressing complex biological issues such as the evolution of phenotypic integration it is of course impossible to address all relevant causal factors within a single study (especially because intra- and inter-population phenomena will inevitably tend to be confounded),

but the following summary should help focus on our expectations and interpretation of the results obtained, as well as aid in outlining follow-up studies given the hypotheses we were not able to discard. 1) Evolution of trait means and plasticities. This could occur because of historical non-deterministic processes such as genetic drift and migration, by differentiating selection (i.e., when different reaction norms are favored in different populations), or by homogenizing selection (when similar reaction norms are favored in different populations). 1A) In the case of *drift* we would expect the populations to be differentiated for their reaction norms, but with no relationship between the shape of reaction norms and reproductive fitness; we would also not expect to find a relationship between reaction norms and habitat of origin in these populations. The first two predictions are testable using our data, the latter is testable in principle, but we do not have the relevant information yet. 1B) *Differentiating selection* would yield different reaction norms in different populations (as in the case of drift), but one would also expect a relationship between reaction norms and reproductive fitness (Pigliucci and Schlichting 1996); there should also be a correlation between the shape of the reaction norm and the geographic provenance of our accessions. Again, the latter expectation is not testable with our data, but the possibility of a relationship between reaction norms and fitness would discriminate between drift (no relationship expected) and differentiating selection (relationship predicted). 1C) *Homogenizing selection* would yield no differentiation in the reaction norms of our populations and consequently no possible relationship with habitat of origin. The prediction of no differentiation is enough to separate this latter hypothesis from the previous two. 1D) A final alternative is for reaction norms not to be able to differentiate because of *genetic constraints*, namely the lack of genetic variation for trait means or plasticities (see, e.g., Barton and Partridge 2000; Camara and Pigliucci 1999; Mitchell-Olds 1996b). This possibility would not be easy to distinguish from homogenizing selection. Of course, both above and in the next set of scenarios, more than one process may have

occurred simultaneously, but our classification will help keeping the different possibilities distinct and identifying which one, if any, was the dominant factor explaining the observed patterns.

2) Evolution of character correlations (phenotypic integration). The same four causes (homogenizing or differentiating selection, constraints and drift) could of course be affecting the evolution of character correlations and how they respond to environmental changes. In particular: 2A) Selection may favor environment-specific sets of correlated characters and, if genetic constraints (depending on the genetic variance-covariance matrices of the populations) did not pose limits to adaptive evolution, we expect to see significant differences in the multivariate structure between environments (the question of *which* sets of co-varying traits one would expect under different hypotheses is explored in a separate paper using a different data set: Pigliucci, in press). 2B) Selection of the kind just described, however, could be halted or slowed down by the existence of strong pleiotropic effects linking the expression of the same trait in different environments (Andersson and Shaw 1994; Eisen and Saxton 1983); in such case, we would predict no or little differentiation of the multivariate structure between environments. 2C) However, physiological trade-offs might change between environments (e.g., Galen 2000; Shirley and Sibly 1999; Tatar and Carey 1995), which would again yield significant differences in trait covariances under different treatments. 2D) Alternatively, selection might favor the same covariance sets in the range of light environments we tested and—if constraints or trade-offs act along the same direction—yield again no or little differentiation of the multivariate phenotype between treatments. 2E) Homogenizing selection could also be precluded by the genetic variance-covariance matrices or by trade-offs, which might result in significant differences in trait covariation between environments. 2F) Finally, drift might have been the dominant force altering the environment-specific expression of trait covariation. It is difficult to predict what this would do, but by analogy with the case of multivariate evolution under drift discussed by Lande (1979; see also Roff 2000) it is plausible to expect identity or proportionality of the correlation matrices

measured in the different environments. Clearly, the number of alternatives here is fairly large, and several possibilities lead to the same predictions (Travisano et al. 1995). This may be due to the fact that we have a much poorer understanding of the forces driving the evolution of character correlations (Camara et al. 2000; Klingenberg et al. 2001; Mezey et al. 2000; Wagner and Schwenk 2000; Wolf et al. 2001). Without more detailed knowledge of the costs and benefits of specific phenotypic syndromes in *A. thaliana* (e.g., Dorn et al. 2000) it is difficult to discriminate among several of the above listed hypotheses, which is a rather typical situation in ecology and certainly does not imply that we cannot make progress in this area.

Materials and Methods

Bulk collections of seeds from 40 populations of *Arabidopsis thaliana* (L.) Heynh. were obtained from the *Arabidopsis* Information Management System (AIMS at http://aims.cps.msu.edu/aims/, where additional information can be obtained about the geographical location and climate of each accession): CS0911, Estland (Germany); CS0913, Petergof (Russia); CS0915, Wassilewskija (Russia); CS0916, Condara (Tadjikistan); CS0917, Darmstadt (Czechoslovakia); CS0920, Enkheim (Ukraine); CS0922, Hodja-Obi-Garm (Tadjikistan); CS0925, Litvania (Litvania); CS1184, Gudow (Germany); CS1214, Guckingen (Germany); CS1226, Hilversum (Netherlands); CS1240, Isenburg (Germany); CS1252, Vranov (Czechoslovakia); CS1282, Rodenbach (Germany); CS1284, Koeln (Germany); CS1504, Seis (Italy); CS1514, Slavice (Czechoslovakia); CS1604, Wietze (Germany); CS1630, Wildbad (Germany); CS1637, East Malling (UK); CS1640, Tsu (Japan); CS1643, Oystese (Norway); CS3109, Copenhagen (Denmark); CS3110, Weiningen (Switzerland); CS3179, Graz (Austria); CS3180, Coimbra (Portugal); CS6003, Koln (Germany); CS6016, Maidstone (UK); CS6023, Sedmouth (UK); CS6034, Bretagny (France); CS6036, Bretagny (France); CS6038, Kelsterbach (Germany);

CS6041, Kelsterbach (Germany); CS6046, Koln (Germany); CS6068, Kent (UK); CS6105, Kelsterbach (Germany); CS6187, Washington (USA); CS6194, Blanes (Spain); CS6195, Wurzburg (Germany); and CS6682, Dijon (France).

All families selected for our experiment represented early flowering populations of *A. thaliana* and had been bulk propagated at AIMS to maintain genetic variation. In order to minimize maternal effects and increase seed availability, we grew the material for one generation under controlled laboratory conditions of 16/8 hour of light/darkness at a room temperature of 23-25°C and provided bottom watering every other day (to minimize mechanical interference). Seeds were then collected, stored in a dry place for 8 weeks and used in the experiment.

These second-generation seeds were placed on a wet filter paper and cold-treated for a week at 5°C in a refrigerator. Imbibed seeds were then transferred to a standard pro-mix soil and placed under high intensity light racks under conditions similar to above. Seedlings were randomly thinned after five days, leaving one plant per 4cm by 4cm by 4.5cm pot. Plants were bottom watered every other day with no addition of fertilizer. We applied two treatments, differing in the level of light intensity: *medium light*, with a photon flux of 240 $\mu M/m^2/sec$, and *low light*, with a photon flux of 70 $\mu M/m^2/sec$ (medium and low refers to a comparison with actual conditions in the field experienced by this species: Callahan and Pigliucci in press).

Measurements

We measured three sets of traits: vegetative, architectural and reproductive. This ensemble constitutes a standard set of characters summarizing major aspects of *A. thaliana*'s phenotype, as discussed in previously published papers (e.g., Pigliucci and Schlichting 1995, 1996, 1998). The *vegetative traits* were quantified at the bolting stage, when the rosette begins to produce the flowering stem: 1) Rosette leaf number, quantifying meristem allocation to vegetative growth. 2) Specific leaf area (calculated as leaf area/leaf weight), for which we

measured the 5th or 6th leaf at the time of the opening of the first flower. This leaf was removed from the plant, pressed, and a picture was taken using a digital image analysis system running Image-Pro™ software; the area was measured using the digitized picture. 3) Leaf chlorophyll content, measured on a fresh leaf, immediately after its removal from the rosette, using a Minolta Chlorophyll Meter (Spad-502) and standardized as chlorophyll content/leaf weight (it was not possible to use dry weight given the very small leaves produced by these plants). *Plant architecture* traits were measured during or after the reproductive phase: 4) Length of the main stem (plant size) and 5) Number of lateral branches (plant architecture). *Reproductive traits* were measured after plants set the first fruits: 6) Time of first reproduction, when the first seeds matured and the siliques started opening, counted as days from bolting (i.e., from the beginning of the reproductive phase); and 7) Total fruit production (reproductive fitness).

Experimental design and statistical analysis

Plants from each family were randomly assigned to one of the two treatments (low or medium light), with every family represented by six replicates within each treatment. The total size of the experimental population was therefore 40 families by 2 treatments by 6 replicates = 480 plants. Individuals were placed in two growth racks equipped with high intensity lights. Each rack housed three shelves, with two trays on each shelf. Each tray contained one replicate of each family, yielding 40 individual plants per tray.

Measured variables deviating from normality or homoscedasticity were appropriately transformed (Sokal and Rohlf 1995). We employed a nested mixed-model analysis of variance (split-plot design: SYSTAT 2000) to estimate the significance of the following factors: A. Population, testing for genetic variation in character means independently of the environment. B. Treatment, estimating overall phenotypic plasticity independent of population effects. C. Population by Treatment interaction, testing for the existence of genetic differentiation for

plasticity among populations. D. Tray (nested within Treatment), estimating the degree of micro-environmental variation due to the experimental setup. Treatment was considered a fixed effect, while Population was treated as a random effect. According to Sokal and Rohlf (1995), if the Tray effect were significant, then the Treatment effect was tested over Tray. Also, if the Population by Treatment interaction showed a significant effect, we tested Population over the interaction term. Otherwise, factors were tested over the error mean square (this judicious use of conservative statistical tests is advocated by Sokal and Rohlf, and we consider it better than always testing over interaction or lower-level effects, even when these are not significant). Given the high number of multiple comparisons (several traits), we used a sequential Bonferroni correction to adjust the nominal α-values for the ANOVAs across rows in Table 1 (again, this correction is moderately conservative, as opposed to a straight Bonferroni, which tends to overcorrect for type II errors: Rice 1989). We then plotted mean reaction norms for all traits with a significant Treatment or Population by Treatment term.

To explore the relationship between environmentally-variable character expression and reproductive fitness we used regression analyses investigating the presence of linear and/or quadratic relationships (Lande and Arnold 1983) between each measured trait and total fruit production. Since our interest was on environmental effects, for this analysis we only considered the characters that showed either a significant Treatment or Treatment by Population interaction. The regressions were conducted separately for the two treatments.

We also calculated treatment-specific correlation matrices and ran standard principal components analyses on them to visualize the sets of covarying traits in each environment as well as how differentiated the multivariate phenotype of our populations was. The variance-covariance matrices for the same traits were then subjected to a more rigorous test of similarity by using Common Principal Components (CPC) analysis as implemented in software provided by Patrick Phillips (available at http://www.uoregon.edu/~pphil/). CPC analyses compare the structure of

two or more covariance matrices in a hierarchical fashion, testing for a series of hypotheses including full equality, proportionality or partially shared eigenstructure among matrices (Phillips and Arnold 1999; Steppan 1997). The tests can be carried out in two alternative ways: the *step-up* and the *jump-up* approaches. The 'step-up' method compares pairs of hypotheses assuming increasing similarity among matrices, starting with no relation between matrices against the hypothesis of 1 CPC; it then proceeds to 1 vs. 2 CPCs, and so on (until n-2 common principal components, because the n-1 test is constrained by the total number of degrees of freedom). The last test is of the hypothesis of complete equality vs. proportionality among matrices. The 'jump–up' approach proceeds up the same hierarchy, but each test is against the null hypothesis of no relation among matrices. Phillips and Arnold (1999) recommend use of the jump-up approach because it is easier to interpret from a biological standpoint, although the two procedures tend to yield very similar results.

Results

Genetic, environmental and genotype-environment interaction in individual traits

The analyses of variance showed that all traits except specific leaf area were highly variable among populations (Table 1, see Table 4 and 5 in the Appendix I for a complete table of character means by population and treatment). Specific leaf area was also the only character that was plastic when averaged among populations (main effect of the environment). Only one trait, leaf number at bolting, showed a significant Population by Treatment interaction (i.e., genetic differentiation for plasticity). All traits except the reproductive ones showed significant micro-environmental variation (Tray within Treatment effect).

Reaction norms were plotted for the traits showing a significant Treatment effect or Treatment by Population interaction. Leaf number (Genotype by Treatment effect only) did not

change overall between treatments, with approximately the same average leaf production under medium and low light (Figure 1a). This is interesting because it shows invariant production of leaves across a substantial change in photosynthetically useful radiation (the medium intensity treatment had three and a half times the light of the low intensity treatment). It is important to note that leaf number is also usually considered as an alternative indication of flowering time in *Arabidopsis*, because these plants normally start flowering when leaf production is halted (Alonso-Blanco et al. 1998. Indeed, these two traits were highly correlated in our experiment). The significant G by T effect was evident in extensive crossing of the individual populations' reaction norms, with some (e.g., CS6041) producing fewer leaves under low light and others (e.g., CS3180) increasing leaf production under the same conditions. The overall variation in leaf number was also remarkable, with CS913 producing only 4-6 leaves and CS916 yielding as many as 18-19.

The specific leaf area (Environment effect only) showed a definite trend toward increase under low light levels (Figure 1b), a potentially adaptive reaction to compensate for lower availability of photosynthetically active radiation. While the overall difference among lines was again substantial (compare CS917 with CS6023, with ratios ranging from 37 to 91 under low light), it was also clear that at least one line (CS917) reacted in a diametrically opposite fashion by actually reducing its SLA under low light. This, together with the observation of lines that were essentially unresponsive (e.g., CS6194), suggests the existence of some biologically meaningful genotype by treatment interaction, despite the lack of statistical significance which may have been due to the overwhelming number of lines with essentially parallel norms of reaction for SLA.

Effects of plastic traits on reproductive fitness

To explore the effects, if any, of the plastic traits (i.e., those showing either overall plasticity or genotype by environment interaction) on reproductive fitness, we conducted a series of linear and quadratic regression analyses (Table 2). Under medium light we found a negative relationship between both leaf number and SLA and reproductive fitness. No quadratic effects were significant under medium light.

The results under low light were somewhat different (Table 2). We found a significant negative relationship between reproductive fitness and specific leaf area, as well as a significant instance of correlational selection on combinations of SLA and leaf number. The pattern (not shown) was one of high reproductive fitness associated with either a large SLA and small leaf number, or with a large leaf number and a small SLA. The plants with lowest fitness had combinations of either low SLA and low leaf number, or high SLA and high leaf number.

Multivariate phenotype and phenotypic integration

The Pearson correlation coefficients among traits followed the same pattern in the two environments, though in some cases correlations of similar magnitude were significant in one treatment but not the other; most correlation coefficients were relatively low in magnitude (Table 3). In both treatments we found a negative correlation between chlorophyll and leaf number, a positive one between main stem length and fruit production, a negative relationship between chlorophyll and main stem length, a positive one involving number of branches and fruit production, and a negative correlation between chlorophyll and fruit production.

Visual inspection of the principal components' vectors showed that the first two principal components had very similar structure between the two treatments, with the loadings of the original variables on the first two principal components being essentially identical to each other (Figure 2). In general, chlorophyll and SLA were positively related to each other and negatively

related to leaf number and main stem length. Lateral branching and fruit production clustered together and independently of most other traits, while first reproduction identified a vector of its own in PC1-PC2 space, falling between chlorophyll-SLA and branching-fruits.

The Common Principal Components analysis also indicated a high stability of the covariance matrix across treatments. Using the jump-up approach we found that the variance-covariance matrix expressed under low light was proportional to the one expressed under medium light (test of equality vs. unrelated hypotheses: $\chi^2 = 43.92$, d.f. = 28, $p = 0.0283$, thereby rejecting equality).

Discussion

Phenotypic evolution is a complex field of study that involves an understanding of the amount of variation for characters, of their lability to environmental conditions, their association with fitness, as well as their relationships with other aspects of the phenotype (Schlichting and Pigliucci 1998). Most studies of reaction norms are conducted within populations of organisms with the intent of uncovering the amount of genotype by environment interaction present (Scheiner 1993), and therefore to estimate the responsiveness of character means and plasticities to natural selection (Via 1987).

Here, we attempted to characterize phenotypic divergence among populations and to study how the correlations among traits are affected by changes in an important component of the environment, light quantity. As Armbruster and Schwaegerle (1996) have pointed out, inter-population studies give us a picture of the outcome of recent evolutionary events leading to differentiation, rather than of potential response to environmental forces acting in the future. This intermediate level of analysis is important to bridge population biology with macroevolution at

and above the species level, as yet an elusive goal of modern evolutionary theory (Hansen and Martins 1996).

While we investigated individual traits and their relationship with reproductive fitness, we were also interested in multivariate patterns of phenotypic integration and their lability to environmental change (Schlichting 1989). There is a considerable interest in the study of phenotypic and genetic correlations because of their relevance to evolutionary theory (Roff 2000; Shaw et al. 1995; Turelli 1988), but few of these studies are carried out at the across-population (or species) level. This is a rather peculiar situation, given the fact that multi-level studies have been carried out on other aspects of phenotypic evolution such as allometric relationships, which have long been studied within population, among populations, ontogenetically, and across species (Ackerly and Donoghue 1998; Gould 1966; Klingenberg and Zimmermann 1992; Pigliucci et al. 1996). It is this complex set of relations between traits, their plasticities, and their correlations that we wanted to explore in this study in the case of *Arabidopsis thaliana*, a model system used in molecular and organismal biology. Of course, a major shortcoming of this and other papers published so far on *A. thaliana* is the paucity of information about the autoecology of this species (Pigliucci 1998), which makes it difficult to interpret results obtained under controlled conditions (a situation which is in the course of being remedied because of recent ongoing efforts of several investigators in the *Arabidopsis* community). On the other hand, it is reasonable to proceed on the assumption that the behavior of the plant under controlled but realistic conditions is not entirely decoupled from what the species does under field conditions. This particular assumption has been tested and validated in our lab in the case of response to light availability in *A. thaliana* (Callahan and Pigliucci in press).

What drives the evolution of trait means and plasticity to light quantity in A. thaliana*?*

In our experiment we have observed widespread genetic variation for across-environment trait means among accessions of *A. thaliana*. By contrast, only one trait was significantly plastic, and only one showed genetic differentiation for plasticity across accessions. These results suggest that there is more genetic differentiation for character means among populations of *A. thaliana* than variation for plasticity to light. Referring back to the scenarios laid out at the end of the Introduction, the former outcome is consistent with a mix of drift and differentiating selection, given that several, but not all, traits were significantly correlated with reproductive fitness. The almost inexistent variation for plasticity across populations is instead more compatible with the hypothesis that homogenizing selection or strong genetic constraints have precluded these populations from diverging in the patterns of their plastic responses.

Such tentative conclusions are consistent with this species' life history: *A. thaliana* is a colonizer of mostly open habitats, where there is little or no shade (Callahan and Pigliucci in press). This sort of environment is coarse grained and is expected to promote genetic differentiation, not plasticity (Bell and Reboud 1997). While *A. thaliana* does show a well-defined shade avoidance response manifested as hypocotyl and stem elongation and accelerated phenology under shade (Ballare` 1999; Callahan et al. 1999), this may be an evolutionary leftover from recent ancestors, especially in light of mixed results concerning the actual adaptive significance of shade avoidance in *A. thaliana* (Dorn et al. 2000). A more stringent test of the drift vs. selection scenario will have to wait for work in progress on characterizing the environment of specific populations and attempts to relate that information to specific functional hypotheses concerning the phenotypic architecture of these plants (J. Dole, M. Cruzan, and M. Pigliucci, in prep.).

The ability to appropriately respond to environmental conditions has been linked to habitat expansion in other species of Brassicaceae. For example, Byers and Quinn (1998) suggest

that the expansion of *Alliaria petiolata* in a broad range of habitats in New Jersey is related to phenotypic plasticity in response to moisture and light availability. The little plasticity and variation for plasticity to light availability found here contrasts with marked responses of *A. thaliana* to other factors, such as water (e.g., Pigliucci et al. 1995; Yamaguchi-Shinozaki et al. 1995) and nutrients (e.g., Pigliucci and Schlichting 1995; Sills and Nienhuis 1995). This difference is also probably a reflection of the ecology of this plant. While light quantity is a coarse grained aspect of the environment for *A. thaliana*, this species experiences dramatic differences in water and nutrient availability over very short spatial and temporal scales (Stratton and Bennington 1996). Differences in the grain of distinct components of the environment thus may promote a balance between genetic differentiation for character means and phenotypic plasticity, depending on which environmental factors one is considering.

Proper caution in interpreting results in an adaptive fashion notwithstanding (Pigliucci and Kaplan 2000), it is interesting to note that both traits that showed either plasticity or genetic variation for plasticity were expressed during the vegetative part of the life cycle, i.e., when low light more dramatically impacts the plant's phenotype. Plants grown under low light consistently produced larger leaves per unit of weight, a response that seems unlikely to be explained simply as a passive reaction caused by lower growth rate, and which is consistent with functional ecological considerations (an increase in leaf area should be particularly advantageous under reduced photosynthetically active radiation). Interestingly, however, in our experiment specific leaf area turned out to be *negatively* associated to fruit output even under low light. This apparently contradictory result may be explained by the trade-off (statistically significant only under low light) between leaf production and SLA so that plants had two alternative strategies to adopt: either increased leaf production but decreased specific area, or vice versa. It will be interesting to explore how common this trade-off is in *A. thaliana* and some of its close relatives,

as well as to find out if particular local environmental conditions select for one or the other strategy.

Investigations of the purported adaptive significance of plastic responses have been carried out in only a few other cases. For example, Dudley and Schmitt (1996) successfully showed the context-dependent fitness effects of stem elongation by phenotypically manipulating *Impatiens capensis*, thereby confirming the adaptive plasticity hypothesis for at least one component of shade avoidance. Weinig (2000) found similar results in *Abutilon theophrasti*, also concerning stem elongation and shade avoidance. On the other hand, Winn (1999) rejected the adaptive plasticity hypothesis for heterophylly (variation in leaf shape) in *Dicerandra linearifolia*. In general, as Sultan (1995) correctly pointed out, testing adaptive plasticity hypotheses requires a better and subtler knowledge of the ecology of a species than it is often available. In general, part of the reason why only some traits may respond in a more or less clearly functional fashion may be that plants react to multiple environmental changes simultaneously at different levels, from physiological to phenological, to morphological responses. Sultan (1995), for example, studied the physiological response of four species of *Polygonum* to complex environments in which light quantity, moisture and nutrients varied in combination. She found a striking difference in the way these species react to changes in the environment, with two taxa maintaining high levels of photosynthetic rates when switched from low to high light and the remaining two taxa dropping their rates dramatically during the same transition. The interspecific differences reflected the differential range of natural ecological conditions under which these plants are found (Sultan 1995).

What drives the evolution of phenotypic integration in A. thaliana*?*

While variation in single characters has been the classical focus of population and evolutionary biology, increasing attention has been granted to the co-variation among characters,

usually referred to as "phenotypic integration" (Armbruster and Schwaegerle 1996; Merila and Bjorklund 1999; Schlichting and Pigliucci 1998). We are particularly interested in the relationship between phenotypic integration and environmental variation, i.e., in how the environment can alter the patterns of phenotypic and genetic correlations among traits. Aastveit and Aastveit (1993) have explained why genotype by environment interactions result in very different estimates of character correlations in different environments and several authors have demonstrated that this is indeed what happens in a variety of organisms (e.g.: Newman 1994; Waitt and Levin 1993; Windig 1994).

Our experimental set up can tell us much less about the evolution of character correlations and their environmental lability in this species given the number of possible scenarios outlined in the Introduction. Consistently with Aastveit and Aastveit's expectations, our results show that light quantity did not significantly alter the structure of the character correlation matrix, in accordance with the little G by T observed for individual traits. A qualitative inspection of the reduced structure of the matrix across environments in our accessions by means of a standard principal components analysis did not reveal any major rearrangement of the vectors summarizing the orientation of traits in multivariate space. Furthermore, explicit tests using common principal components analysis failed to reject the hypotheses of equality or proportionality (depending on which subset of data was used) of the two matrices. Of course, this outcome may be explained by a variety of processes, including selection favoring environment-specific covariance sets of characters being counterbalanced by strong inter-environment pleiotropic effects (Schlichting and Pigliucci 1993; Via 1993), selection favoring the same covariance sets across environments reinforced by existing constraints or physiological trade-offs, and perhaps the simple action of genetic drift. Our findings are by no means typical, however, as marked environmentally-induced changes in the genetic architecture of natural populations have been documented before (e.g., Bell 1992; Service and Rose 1985; Simons and Roff 1996),

including a case for *Arabidopsis* in response to nutrient availability (Pigliucci and Schlichting 1998).

Concluding remarks

We have shown that reaction norms to light quantity of populations of *Arabidopsis thaliana* display a high degree of genetic differentiation but little plasticity or genotype by treatment interactions. However, at least one plastic trait displays a complex relationship with reproductive fitness which is consistent with the combined effect of natural selection and tradeoffs mediated by resource limitation. Furthermore, the environmental lability of the trait correlation matrix across these accessions is also very limited, as predicted in cases of little genotype-environment interactions. We conclude that these populations have genetically diverged over the course of their recent evolutionary history while maintaining a general invariance to light quantity, probably because of a combination of homogenizing selection and genetic/physiological constraints.

The scenarios outlined in the Introduction, among which we were able to discriminate only partially, could be further examined by collecting information on the local habitat of origin of our accessions (confirming or rejecting the drift vs. differentiating selection vs. homogenizing selection hypotheses). In order to gain more discriminatory power among the several possible mechanisms underlying the evolution of character correlations one will need to gain more insight into the basic physiology of *A. thaliana*, certainly something possible given the extensive use of this species as a model system, as well as on its within-population level genetic constraints (Mitchell-Olds 1996a) and selective pressures (Callahan and Pigliucci in press).

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Appendix I.

Table 1. ANOVA table of Mean Squares and p-values for each factor and interaction included in the model. Degrees of freedom are indicated in parentheses in the top row. Values significant after a sequential Bonferroni correction (across traits) are in boldface.

Trait	**Population** *(39 df)*	**Treatment (medium vs. low light)** *(1 df)*	**Population*Treatment** *(39df)*	**Tray(Treatment)** *(11 df)*	**Error** *(282-311 df)*
Leaf number at bolting	97.95 **0.0000**	3.23 0.2433	4.57 **0.0011**	9.22 **0.0000**	2.36
Specific leaf area (Square root)	1.80 0.0917	36.35 **0.0019**	0.85 0.4340	2.19 **0.0032**	0.83
Chlorophyll standardized	2.07 **0.0000**	0.14 0.5211	0.32 0.5881	2.74 **0.0000**	0.35
Main stem length	88.02 **0.0016**	276.24 0.0830	33.47 0.0542	75.99 **0.0003**	23.47
Lateral branches	4.72 **0.0002**	0.06 0.8287	1.43 0.1956	2.98 **0.0047**	1.18
First reproduction	38.90 **0.0120**	172.34 0.0573	18.66 0.3845	38.26 0.0160	17.65
Total fruit	1117.82 **0.0093**	2047.69 0.1431	518.80 0.4501	823.49 0.0943	511.00

Table 2. Regression analyses of characters showing plasticity or genotype by treatment interaction against reproductive fitness. Quadratic and interaction terms were investigated once the effects of linear terms were removed. Boldface indicates significance at $\alpha < 0.05$.

		Effect	***Std Coefficient***	***t***	***P(2 Tail)***
Linear terms	*Medium*	Leaf number at bolting (LB)	-0.15	-2.28	**0.0234**
		Specific leaf area (SLA)	-0.24	-3.62	**0.0004**
	Low	Leaf number at bolting	-0.08	-0.98	0.3311
		Specific leaf area	-0.24	-2.90	**0.0043**
Quadratic and interaction effects	*Medium*	LB^2	0.24	0.68	0.5055
		SLA^2	0.17	0.48	0.6352
		SLA×LB	-0.23	-0.68	0.4983
	Low	LB^2	-0.62	-1.37	0.1728
		SLA^2	-0.44	-1.39	0.1662
		SLA×LB	-1.14	-2.24	**0.0266**

Table 3. Pearson correlation coefficients among traits under medium and low light. Boldface indicates significance after a table-wide Bonferroni correction.

Medium Light	Leaf number at bolting	First reproduction	Main stem length	Number of lateral branches	Total fruit	Chlorophyll standardized	Specific leaf area
Leaf number at bolting	1						
First reproduction	-0.05	1					
Main stem length	0.12	-0.17	1				
Number of lateral branches	0.10	0.08	0.21	1			
Total fruit	-0.11	0.14	**0.37**	**0.29**	1		
Chlorophyll standardized	**-0.33**	0.02	**-0.28**	-0.19	**-0.29**	1	
Specific leaf area	-0.19	0.02	-0.15	-0.04	**-0.21**	**0.25**	1

Low Light	Leaf number at bolting	First reproduction	Main stem length	Number of lateral branches	Total fruit	Chlorophyll standardized	Specific leaf area
Leaf number at bolting	1						
First reproduction	-0.19	1					
Main stem length	**0.28**	-0.16	1				
Number of lateral branches	0.14	0.23	**0.35**	1			
Total fruit	-0.06	0.06	**0.41**	**0.53**	1		
Chlorophyll standardized	**-0.25**	-0.07	**-0.37**	**-0.33**	**-0.39**	1	
Specific leaf area	-0.05	0.06	-0.17	-0.08	-0.20	0.24	1

Table 4. Accession means for traits measured under Low Light**.**

Accession	*Leaf No at bolting*	*First reprod.*	*Main stem length*	*Lateral braches No*	*Total fruit*	*Chlorophyll Stand.*	*SLA*
CS0911	7.50	23.50	20.05	1.75	33.50	1.66	8.10
CS0913	4.80	26.60	24.94	2.00	59.80	1.28	9.31
CS0915	5.60	26.60	19.58	2.40	35.20	2.17	9.36
CS0916	19.00	19.33	24.93	2.00	34.33	0.50	8.01
CS0917	8.00	22.00	19.40	1.80	44.00	1.84	6.26
CS0920	9.00	20.50	26.95	2.50	39.25	1.35	8.19
CS0922	13.00	22.00	28.42	1.67	42.67	0.63	7.99
CS0925	11.33	20.00	23.67	1.33	30.67	1.41	7.90
CS1184	9.00	22.20	24.46	2.00	35.60	1.19	8.12
CS1214	8.00	29.75	19.43	2.50	55.50	0.75	8.72
CS1226	8.00	26.75	21.40	4.00	55.75	1.18	8.63
CS1240	10.80	21.80	25.40	2.80	43.20	1.56	8.70
CS1252	7.00	27.25	24.88	1.75	26.75	2.05	8.81
CS1282	13.50	21.00	28.05	2.75	39.75	0.65	7.97
CS1284	8.67	24.67	22.58	1.83	23.33	1.98	9.19
CS1504	7.83	25.50	26.98	2.67	30.67	1.57	8.15
CS1514	6.00	23.20	28.08	3.00	47.80	1.15	7.87
CS1604	13.33	25.67	38.00	4.67	34.00	1.47	9.02
CS1630	15.00	24.40	31.12	3.40	49.20	0.71	7.79
CS1637	18.50	25.50	28.50	2.50	23.50	1.08	8.19
CS1640	18.33	23.33	30.23	3.33	23.00	0.81	8.50
CS1643	12.33	24.83	21.65	2.83	45.00	1.39	8.24
CS3109	12.50	22.00	25.88	3.25	15.50	1.96	9.34
CS3110	7.50	29.67	19.77	3.17	25.33	1.20	8.01
CS3179	14.50	24.00	25.25	3.50	24.00	1.27	8.79
CS3180	17.50	20.50	21.15	2.50	18.00	1.22	8.39
CS6003	12.50	26.00	30.10	4.50	76.00	1.00	7.98
CS6016	15.75	23.00	25.08	2.75	19.75	1.72	8.49
CS6023	12.00	22.00	23.25	2.25	27.25	0.67	9.46
CS6034	7.20	21.60	25.48	1.40	23.20	1.13	8.09
CS6036	13.33	24.67	30.33	1.67	14.33	1.08	8.77
CS6038	9.33	24.67	27.08	2.50	38.50	1.35	7.88
CS6041	10.00	22.83	29.30	2.83	33.83	1.28	8.86
CS6046	11.00	22.25	28.48	3.50	59.00	1.12	8.36
CS6068	13.00	25.00	29.00	4.00	37.00	0.81	7.17
CS6105	8.33	20.50	21.98	2.17	27.67	1.48	8.68
CS6187	16.00	19.50	28.30	2.00	38.50	0.91	8.17
CS6194	13.33	22.00	31.20	3.67	62.67	0.83	7.19
CS6195	14.00	20.75	31.25	3.50	57.25	1.16	7.91
CS6682	8.80	22.00	26.18	1.00	18.60	3.91	7.98

Table 5. Accession means for traits measured under Medium Light.

Accession	*Leaf No at bolting*	*First reprod.*	*Main stem length*	*Lateral branches No*	*Total fruit*	*Chlorophyll stand.*	*SLA*
CS0911	8.50	17.33	17.23	1.67	37.83	1.61	7.19
CS0913	6.00	22.00	26.57	2.17	47.50	1.33	7.43
CS0915	7.00	22.67	21.88	2.67	58.00	1.90	7.65
CS0916	18.80	16.60	24.62	1.40	37.00	0.21	7.14
CS0917	8.17	18.00	21.03	2.17	58.33	1.16	7.56
CS0920	9.00	18.50	26.80	2.83	42.83	1.16	7.05
CS0922	12.17	18.33	26.35	2.00	51.67	0.80	6.77
CS0925	11.00	20.83	23.07	3.17	39.50	1.28	7.58
CS1184	9.00	17.83	25.75	1.33	41.17	1.40	7.13
CS1214	9.00	21.00	19.75	3.67	52.67	1.26	7.50
CS1226	8.17	21.50	21.50	3.50	61.83	1.56	7.22
CS1240	11.17	19.00	17.12	2.33	28.83	1.65	7.33
CS1252	7.50	20.00	20.23	1.67	31.50	1.30	7.39
CS1282	13.00	20.17	21.40	3.33	42.50	0.61	6.97
CS1284	8.00	19.00	25.13	3.00	42.33	2.12	7.25
CS1504	7.80	19.00	24.78	3.20	36.80	1.24	7.06
CS1514	5.83	21.67	25.00	2.83	85.17	0.94	6.82
CS1604	14.17	25.50	29.87	3.83	48.00	1.33	6.99
CS1630	15.67	20.50	23.12	3.50	39.17	1.09	7.04
CS1637	15.33	17.83	24.75	2.33	46.33	0.81	6.39
CS1640	17.17	19.83	25.77	2.50	37.50	0.69	6.83
CS1643	12.33	26.67	14.95	1.83	42.17	1.53	7.31
CS3109	11.17	17.50	20.82	2.00	56.67	1.19	6.75
CS3110	8.17	20.00	21.12	2.83	35.00	1.45	6.97
CS3179	11.33	19.00	23.80	2.00	47.33	1.20	7.48
CS3180	14.50	17.83	20.27	2.83	42.33	0.97	7.59
CS6003	12.83	18.50	20.90	2.33	31.50	1.36	6.74
CS6016	12.40	19.40	19.52	3.40	47.00	1.25	6.73
CS6023	9.17	24.33	19.50	2.00	47.83	1.37	7.59
CS6034	9.00	18.83	22.80	2.50	52.33	1.52	7.02
CS6036	13.17	18.00	26.63	1.67	22.33	1.01	7.72
CS6038	9.60	20.00	24.42	2.00	50.60	0.96	6.79
CS6041	12.50	22.33	20.83	3.50	39.00	1.75	7.40
CS6046	11.00	18.67	20.38	3.17	51.17	0.91	7.17
CS6068	13.33	20.50	24.25	3.17	50.83	1.06	6.78
CS6105	10.00	18.67	25.02	3.00	55.00	0.99	6.80
CS6187	14.33	17.83	25.62	3.67	63.17	0.77	6.52
CS6194	11.80	18.80	22.24	3.40	59.60	1.07	7.34
CS6195	12.17	18.33	24.85	3.83	65.67	1.61	7.14
CS6682	9.80	21.80	20.04	0.60	23.00	2.84	7.08

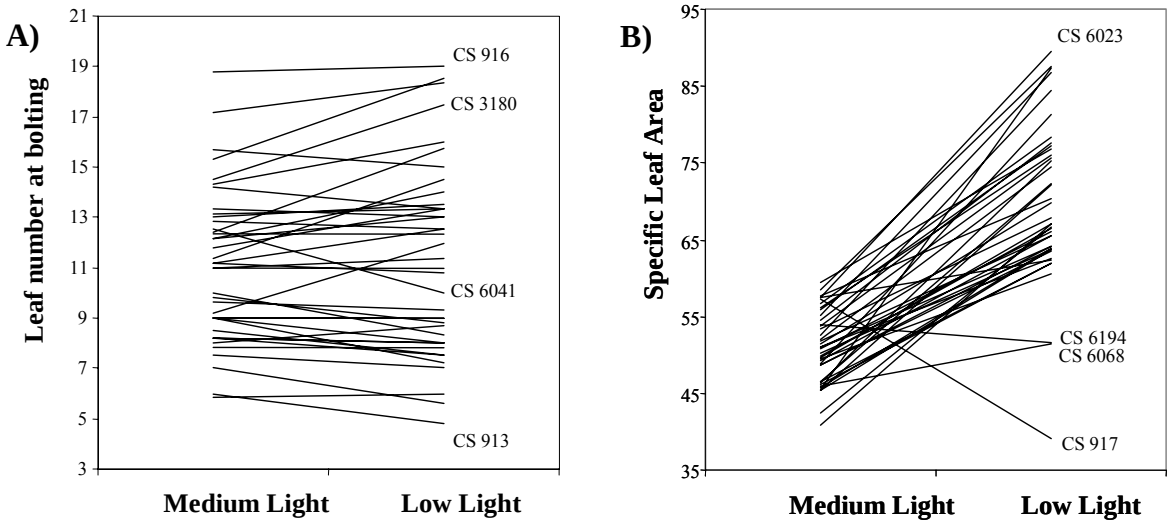
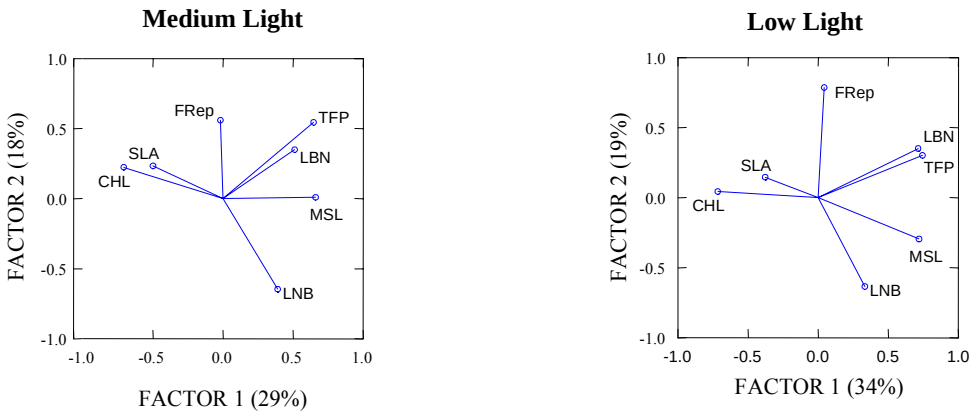


Figure 1. Reaction norms of the traits that showed either a significant treatment effect (overall phenotypic plasticity) or a significant genotype by treatment interaction. Each line represents an individual accession; some lines specifically discussed in the text are highlighted. A) Leaf number at bolting (significant genotype by treatment term); B) Specific leaf area (in mm^2/g, significant treatment term).



Abbreviations used: **Chl** – standardized chlorophyll content of the 5th leaf, **FRep** - first reproduction, **LNB** - leaf number at bolting, **LBN** - lateral branches number, **MSL** - main stem length, **SLA**- specific leaf area, **TFP** - total fruit production.

Figure 2. Plots of vectors quantifying the relationships between each measured variable in the experiment and the first two principal components (factors) calculated for all populations under medium (left) and low (right) light. The length of the vectors is proportional to the intensity of the correlation between each variable and the principal components; the angles of the vectors estimate the correlations across variables (highly positively related if the vectors are parallel, highly negatively related if the vectors are at 180° from each other). The percentage of variance explained by each principal factor is indicated in parentheses.

PART II:

Phenotypic plasticity and integration in response to flooded conditions in natural accessions of *Arabidopsis thaliana* (L.) Heynh (Brassicaceae)

Statement:

This part of the dissertation was submitted for publication to the Annals of Botany.

All tables and figures are located in the Appendix II.

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Running title: Plasticity and integration in *Arabidopsis*

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Introduction

Plants, as sessile organisms, unavoidably experience fluctuations in their external environment, which under certain circumstances may lead to the evolution of phenotypic plasticity in a variety of traits (Bradshaw, 1972; Pigliucci, 2001). Plasticity is the property of a given genotype to produce different phenotypes depending on the environment (Schmalhausen, 1949). Changing environmental conditions are also known to induce distinct patterns of character correlations (Schlichting and Levin, 1986), although it is not clear to what extent this is a result of natural selection for "phenotypic integration" or of genetic constraints in the form of pleiotropy and/or linkage.

The evolution of patterns of phenotypic covariation is of particular relevance to two major areas of inquiry attempting to link macro and microevolutionary processes (Steppan, 1997). On one hand, quantitative genetics models evolutionary change as the result of natural selection and other forces acting on the genetic architecture of phenotypes. On the other hand, research on evolutionary constraints views phenotypic covariances as the expression of underlying genetic and developmental constraints. As pointed by several authors (e.g., Lande, 1979; Arnold and Wade, 1984; Arnold and Phillips, 1999; Phillips and Arnold, 1999) covariance patterns are fundamental to quantitative models of phenotypic divergence over long periods of time. Thus, if we want to apply microevolutionary models to the understanding of macroevolutionary patterns, we need to verify the extent to which genetic covariation remains constant or proportional across populations or species over macroevolutionary time scales (Lande, 1979; Steppan, 1997). The experimental and comparative study of phenotypic integration, then, becomes a crucial component of evolutionary quantitative genetics.

Water is among the chief environmental changes that induce both plasticity of single traits and of character correlations (Pigliucci et al., 1995b; Conner and Zangori, 1998; Meyer and

Allen, 1999; Barrileaux and Grace, 2000). Water is, of course, a crucial and highly variable abiotic factor for every living organism. For plants, both shortage (drought) and excess (waterlogging or flooding) of water is stressful and the two conditions elicit distinct coping mechanisms (Blom and Voesenek, 1996; Mauseth and Plemons-Rodriguez, 1998; Galen, 2000; Zhang et al., 2000). In the case of water excess, on which we concentrate here, the main problem is lowered oxygen content in the soil, which affects primarily the root system and, as a consequence, above ground growth.

Much attention has been paid to plant responses to flooding (Baruch and Merida, 1995; Rubio et al., 1995; Blom and Voesenek, 1996; Visser and Voesenek, 1996; Youssef and Saenger, 1996; Moog, 1998; Grichko and Glick, 2001), with research focused mostly on flood-tolerant species such as rice, and to some extent *Carex* and *Rumex sp.* However, it is equally important to understand how species that are not flood tolerant cope with the occasional excess of water, e.g., due to heavy rain that can fill up spaces between soil particles and cause anoxia.

We have investigated genetic differentiation of reaction norms to flooding as well as flood-induced changes in patterns of character correlations in a collection of populations of the wild mustard *Arabidopsis thaliana* (L.) Heynh (Brassicaceae). *A. thaliana* is a well established model system for studies in molecular biology and physiology (Meyerowitz, 1989; Meinke, 1994; Convay and Poethig, 1997; Coupland, 1997; Ellis and Elisabeth S. Dennis, 1999), and has recently received much attention from an ecological and evolutionary standpoint (Clauss and Aarssen, 1994; Mauricio and Rausher, 1997; Li and Jun-Ichirou Suzuki, 1998; Pigliucci, 1998).

In this paper we address the following questions: 1. Is there genetic variation for trait means among populations when exposed to a range of water availability which includes flooded conditions? 2. Are there plasticity and variation for plasticity to flooding for the traits of interest? 3. Do plastic traits affect reproductive fitness, suggesting that they may be under natural selection depending on the environmental conditions actually experienced by the plants? 4. Do

environmental changes affect the variance-covariance matrix relating different life history and architectural traits, and if so to what extent?

Materials and Methods

Collections of seeds from 47 accessions of *Arabidopsis thaliana* (L.) Heynh. were obtained from the *Arabidopsis* Information Management System (AIMS at www.arabidopsis.org): CS0911, Estland (Germany); CS0913, Petergof (Russia); CS0915, Wassilewskija (Russia); CS0916, Condara (Tadjikistan); CS0917, Darmstadt (Czechoslovakia); CS0920, Enkheim (Ukraine); CS0922, Hodja-Obi-Garm (Tadjikistan); CS0925, Litvania (Litvania); CS0932 Aberdeen (UK); CS1184, Gudow (Germany); CS1214, Guckingen (Germany); CS1226, Hilversum (Netherlands); CS1240, Isenburg (Germany); CS1252, Vranov (Czechoslovakia); CS1258, Jamolice (Czechoslovakia); CS1282, Rodenbach (Germany); CS1284, Koeln (Germany); CS1504, Seis (Italy); CS1514, Slavice (Czechoslovakia); CS1604, Wietze (Germany); CS1630, Wildbad (Germany); CS1635, Canterbury (U.K.); CS1637, East Malling (UK); CS1640, Tsu (Japan); CS3109, Copenhagen (Denmark); CS3110, Weiningen (Switzerland); CS3179, Graz (Austria); CS3180, Coimbra (Portugal); CS6003, Koln (Germany); CS6004, Maidstone Kent (U.K); CS6015, West Malling, Kent, (U.K); CS6016, Maidstone (UK); CS6023, Sedmouth (UK); CS6034, Bretagny (France); CS6036, Bretagny (France); CS6038, Kelsterbach (Germany); CS6041, Kelsterbach (Germany); CS6046, Koln (Germany); CS6047, Maidstone, Kent, (U.K); CS6068, Kent (UK); CS6105, Kelsterbach (Germany); CS6187, Washington (USA); CS6194, Blanes (Spain); CS6195, Wurzburg (Germany); and CS6682, Dijon (France). CS6731 Gluckingen, (Germany); and LER, Landsberg erecta.

All accessions selected for our experiment represented early flowering populations of *A. thaliana* and had been bulk propagated at AIMS to maintain genetic variation. In order to

minimize maternal effects and increase seed availability, we grew the material for one generation under controlled laboratory conditions of 16/8 hour of light/darkness at a room temperature of 23-25°C and provided bottom watering every other day.

These second-generation seeds were then placed on a wet filter paper and cold-treated for one week at 5°C in a refrigerator. Imbibed seeds were then transferred to a mix of top soil-coarse sand-turface (2-2-1 by volume) and placed under combined fluorescent and incandescent light on a three-shelf growth rack. Seedlings were randomly thinned after five days, when the first set of true leaves had emerged, leaving one plant per 7cm (diameter) by 5cm (deep) pot. We applied two treatments: non-flooded and flooded. Plants assigned to the non-flooded watering regime were bottom watered every other day, letting the soil saturate with water for two hours, and then drained. The flooded group had water changed every other day and pots were always kept at saturation so that plants were waterlogged at all time during the experiment. Both treatments were top fertilized once a week for 5 weeks with an 11:11:11 (N:P:K) solution in the amount of 2ml to each pot.

Measurements

We measured three sets of traits: vegetative, architectural and reproductive. The *vegetative traits* were quantified at the bolting stage, when the rosette begins to produce the flowering stem: 1) Bolting time (time from seed planting to the beginning of the elongation of the main stem); 2) Rosette leaf number, quantifying meristem allocation to vegetative growth; and 3) Rosette diameter, a measure of plant size at bolting time. *Plant architecture* traits were measured at the time of harvest, one week after maturation of the first silique: 4) Number of lateral branches; 5) Above ground fresh weight (a measure of plant allocation of resources to above ground growth), comprising the rosette plus the main stem and the branches bearing fruits; 6)

Below ground fresh weight (plant allocation of resources to below ground growth); and 7) Total number of basal stems (allocation of resources to secondary meristems). *Reproductive traits* were also measured when plants set the first fruits: 8) Time of first reproduction, when the first seeds matured and the siliques started opening, counted as days from bolting (i.e., from the beginning of the reproductive phase); and 9) Total fruit production (reproductive fitness).

Experimental design and statistical analysis

Plants from each accession were randomly assigned to one of the two treatments (flooded or unflooded), with every accession represented by six replicates within each treatment. The total size of the experimental population was therefore 47 accessions by 2 treatments by 6 replicates = 564 plants. Individuals were placed in two three-level light racks equipped with two fluorescent and two incandescent light tubes/bulbs per level. Each level on each rack housed four trays and contained two replicates of each family randomly assigned to one of the four trays, yielding 94 individual plants per level and 23-24 pots per tray.

Measured variables deviating from normality or homoscedasticity were appropriately transformed (Sokal and Rohlf, 1995). We employed a nested mixed-model analysis of variance (split-plot design: SYSTAT, 2000) to estimate the significance of the following factors: A. Accession, testing for genetic differentiation in character means among accessions independently of the environment. B. Treatment, estimating overall phenotypic plasticity independent of genetic effects. C. Accession by Treatment interaction, testing for the existence of genetic differentiation for plasticity among accessions. D. Tray (nested within Treatment), estimating the degree of micro-environmental variation due to the experimental setup. Treatment was considered a fixed effect, while Accession was treated as a random effect. Following Sokal and Rohlf (1995), if the Tray effect were significant, then the Treatment effect was tested over Tray. Also, if the Accession by Treatment interaction showed a significant effect, we tested Accession over the

interaction term. Otherwise, factors were tested over the error mean square (this balanced use of conservative statistical tests is advocated by Sokal and Rohlf, and we consider it better than always testing over interaction or lower-level effects, regardless of their significance. Our approach compromises between the often diverging criteria of statistical and biological significance). Given the high number of multiple comparisons (several traits), we used a sequential Bonferroni correction to adjust the nominal α-values for the ANOVAs across rows in Table 1 (again, this correction is moderately conservative, as opposed to a straight Bonferroni, which tends to overcorrect for type II errors: Rice, 1989). We then plotted mean (accession) reaction norms for all traits with a significant Treatment or Accession by Treatment term.

To explore the relationship between environmentally-variable character expression and reproductive fitness we used regression analyses investigating the presence of linear and/or quadratic relationships (Lande and Arnold 1983) between each measured trait and total fruit production. Since our interest in this paper focused on environmental effects, for this analysis we only considered the characters that showed either a significant Treatment or Treatment by Accession interaction. The regressions were conducted separately for the two treatments.

We also calculated treatment-specific correlation matrices and ran standard principal components analyses on them to visualize the sets of co-varying traits in each environment as well as how differentiated the multivariate phenotype of our accessions were. We ran a series of correlation tests comparing each eigenvector (in order of variance explained) in one environment with the corresponding one in the other environment to formally test the degree of multivariate similarity between the two matrices. We decided not to use common principal components analysis (CPC: Steppan, 1997; Phillips and Arnold, 1999) as advocated by some authors, because on several of our data sets this technique has rejected the hypothesis of any similarity of structure when it was evident by visual inspection and by carrying out alternative tests that the matrices were in fact structurally similar. We think that this is a problem with the sensitivity of the CPC

method, which may have a tendency to reject the null hypothesis too often when the data sets are large enough.

Results

Genetic variation among accessions

The analyses of variance showed that all traits except root fresh weight and basal stems were significantly variable among accessions (Table 1). However, these two traits—together with rosette diameter—were the only ones showing any significant Accession by Treatment interaction, which indicates presence of inter-accession differentiation for plasticity. Bolting day, leaf number at bolting, and time to first reproduction were the only traits not showing a main effect of Treatment (i.e., overall plasticity). Not surprisingly, all of the traits measured showed significant effects due to micro-environmental variation (Tray within Treatment effect).

Reaction norms were plotted for the traits showing a significant Accession by Treatment effect (Figure 1). All three plastic traits had higher values under non-flooded conditions than under flooded ones, in agreement with the intuitive expectation that flood constitutes a stress. However, notice that some genotypes hardly responded to the change in environment, especially in terms of rosette diameter and root weight. The diagrams highlight the reaction norms of the line Landsberg *erecta* for comparison, since this is a laboratory line often used in *Arabidopsis* research.

Effects of plastic traits on reproductive fitness

In order to explore the effect of plastic traits on reproductive fitness, we conducted a series of linear and quadratic regression analyses (Table 2). Under flooded conditions all linear

terms were significant, indicating directional selection for increase in rosette size, shoot weight, root weight (marginally significant), number of lateral branches, and number of basal stems. As far as the quadratic terms were concerned, only shoot fresh weight was statistically significantly associated with reproductive fitness under flood. However, a visual inspection of the trait-fitness relationship (Figure 2) showed that the quadratic term did not actually add anything to the explanatory power of the linear one and that its statistical significance was due to the presence of a few outliers.

When we conducted the same analysis for the data from the non-flooded conditions (Table 2) we found that shoot weight, number of lateral branches, and number of basal branches showed statistically significant linear terms, while rosette diameter and root fresh weight were characterized by significant quadratic terms. Here, the quadratic terms were actually informative since there was a clear peak of higher fitness for intermediate rosette and root size, on each side of which plant's fitness clearly decreased (Figure 2).

Multivariate phenotype

We calculated the correlation matrices among all traits under both environmental conditions (Tables 3 and 4). The matrices appeared very similar at a visual inspection, differing slightly in the magnitudes of some of the correlations. Three correlations were statistically significant under flooded conditions (Table 3) but not in the non-flooded environment (Table 4), all involving fruit set, respectively with bolting time (-0.22), leaf number at bolting (-0.24), and number of lateral branches (0.29). Conversely, four correlations were significant under non-flooded conditions but not so in the flooded environment: three involved later branches and, respectively, root fresh weight (0.24), shoot fresh weight (0.32), and rosette diameter (0.38). The last correlation was between bolting day and rosette diameter (0.66). While the fact that one

correlation is significantly different from zero and another one is not does not necessarily imply that the two correlations are in fact distinct, this appears to be the case in many of these instances when one considers the magnitudes of the same correlation under the two treatments (we did not carry out formal tests because of the high number of pairwise comparisons and the lack of a priori hypotheses about which pairs should be significantly heterogeneous).

In order to see if the environment altered the overall structure of the correlation matrix, we performed a principal components analysis to allow visual inspection of the eigenvectors and a vector correlation analysis to carry out a formal test of the degree of matrix similarity between treatments. Visual inspection of the eigenvectors' (Figure 3) showed that the first two factors (the ones explaining the highest amount of variance) were very similar, the major difference being a change in the magnitude of the vector associated with the time of first reproduction (which explained little of the variance on either component under non-flooded conditions). For the rest, the two plots are essentially mirror images of each other along the second factor. Since the actual direction in multivariate space is arbitrary, this means that most of the relationships among variables were unaffected by the environmental change. A more careful inspection of the loadings, however, did reveal some subtle differences between the two treatments (Table 5), including a dissociation of lateral branches from the main group of traits under non-flooded but not flooded conditions, and a somewhat opposite behavior of basal branches.

A formal correlation analysis comparing vectors (in order of explained variance) between the two treatments showed a high degree of similarity of the first two eigenvectors (Table 6). Notice, however, that the environment did have minor effects on the multivariate structure, as evidenced by the low and not significant correlations between vectors 3-7 and 9.

Discussion

Phenotypic evolution is a complex field of study that involves an understanding of the amount of variation for characters, of their lability to environmental conditions, their association with fitness, as well as their relationship with other aspects of the phenotype (Schlichting and Pigliucci, 1998b). Here, we attempted to characterize phenotypic divergence among accessions of a wild weedy species and to study how the correlations among traits are affected by changes in an important component of the environment, water availability. As Armbruster and Schwaegerle (1996) have pointed out, studies carried out among accessions or populations give us a picture of the outcome of recent evolutionary events leading to differentiation, rather than of potential response to environmental forces acting in the future—typically the target of intra-population variation studies. Such intermediate level of analysis is important to bridge population biology with macroevolution at and above the species level.

We investigated individual traits and their relationship with reproductive fitness, as well as the multivariate patterns of phenotypic integration and their lability to environmental change (Schlichting, 1989). There is a considerable interest in the study of phenotypic and genetic correlations because of their relevance to evolutionary theory (Roff and Mouseau, 1999) especially with regard to the validity of the assumptions embedded in quantitative genetic models of evolutionary change (Turelli, 1988; Pigliucci and Schlichting, 1997) and to our understanding of multivariate phenotypic evolution (Schlichting and Pigliucci, 1998a).

Genetic variation among accessions

We have observed widespread genetic variation for across environment trait means among our accessions of *Arabidopsis thaliana* when grown under contrasting water regimes. We

have also observed widespread phenotypic plasticity (six out of nine traits), while only three traits showed genetic differentiation for plasticity. These results indicate that there was much more genetic differentiation for trait means irrespective of the environment than differentiation for plasticity to water availability among accessions of *A. thaliana*. Similar results were reported by Pigliucci et al. (1995b) in their work on different populations of *A. thaliana*, showing a fair amount of plasticity in the populations studied but lack of genetic differentiation for plasticity. This is consistent with the species' life history: *A. thaliana* flowers in the spring and is probably exposed to random fluctuations in flooding regimes which depend on the local geography. Under these conditions these plants are not expected to evolve adaptive plasticity to water, but rather genetic specialization for whatever water regime they encounter more often (Pigliucci, 2001). In addition to fluctuating rainfall/water level, edaphic conditions are important: sandy soils do not cause much water logging since water can percolate through the sediment with ease, while soils richer with silts or clay are characterized by a slower water drainage (Podbielkowski and Podbielkowska, 1992). Unfortunately, not much is known about the edaphic conditions typical of *A. thaliana* populations, though personal observations (MP) seem to indicate that sandy soils are more often occupied by this species.

It is therefore perhaps not surprising that our accessions did not show specialization for root size (one of the few traits that was not heterogeneous among provenances) and that all were performing poorly under the relatively novel environment of water logging conditions. This implies that the observed plasticity was mostly the result of a passive response to severe stress rather than an adaptive response. Similar results were also obtained by Anderson and Pezeshki (2001) in their work on three bottomland tree species. In their study, all of the species used showed a decrease in root dry biomass when exposed to varying periods of flooding, with the highest decrease elicited by long term flooding.

In general, a high degree of differentiation among accessions for trait means has been

observed in other studies of this species (Pigliucci et al., 1995a; Pigliucci et al., 1995b) and is consistent with the highly selfing mating system of this taxon (Abbot and Gomes, 1987), which leads to high among-population and low within-population genetic variation. It is an open question if the observed differentiation is chiefly the result of historical (drift) or deterministic (selection) phenomena.

Plasticity and fitness

Using regression analysis performed on the traits showing plasticity or genetic differentiation for plasticity (rosette diameter, number of lateral branches, number of basal stems, and shoot and root fresh weight), we detected directional selection for increase of all these traits under the flooded treatment, and directional selection for increase in shoot fresh weight and degree of branching (both lateral and basal stems) as well as stabilizing selection for rosette size and root weight under non-flooded conditions. This indicates that it is advantageous to invest more in aerial structures (especially shoots and branches) regardless of the environment, and no selection on plasticity per se on these traits would be expected. The observed differentiation for plasticity of basal stems, therefore, was unlikely to be the result of past selection in response to varying water conditions. Surprisingly, we could not find many studies of selection on plant plasticity in response to water. The only work available in the literature (Dudley, 1996a; Dudley, 1996b) actually deals with adaptation to dry conditions, and is not therefore particularly informative for our system.

The vast majority of plants under flooded conditions produced very small roots, again indicating that they were under severe stress caused by the anoxic conditions of the soil (since our design never dropped the water level below full soil capacity). Indeed, we have observed that plants grown under flooded conditions not only had much shallower root systems, but were

producing adventitious roots from their hypocotyls, a well known compensation mechanism employed by plants grown in anoxic environments (Armstrong and R. Brandle, 1994). Under non-flooded conditions, root size seemed to have an optimal intermediate level, for plants with both too small and too large root systems were at a clear disadvantage. This is likely due to the fact that too little root growth does not provide enough nutrients and water for normal plant growth, but too much allocation to roots when water is not particularly scarce may be detrimental to above-ground biomass. Also under non-flooded conditions we found stabilizing selection on rosette size, implying the existence of an optimal intermediate value for this trait on either side of which reproductive fitness is lowered if water conditions are normal. This could again be due to the detriment to reproductive structures that may be caused by either too little or too much allocation to vegetative ones.

Finally, we have observed apparent "stabilizing selection" under flooded conditions for shoot weight, but this was more likely an artifact caused by the presence of a few outliers and the linear term of the model was sufficient to explain the observed pattern.

Environment and the stability of the covariance matrix

While classical studies on evolutionary ecology tended to focus on the variation in single characters (with some notable exceptions: Berg, 1960; Clausen and Hiesey, 1960), there has been a recent increase in interest in the co-variation among characters and its consequences for phenotypic evolution (Steppan, 1997; Arnold and Phillips, 1999; Phillips and Arnold, 1999; Waldman and Anderson, 2000). We were particularly interested in the relationship between phenotypic integration (assessed by the pattern of character correlations) and environmental variation, i.e., in how the environment can alter the patterns of phenotypic correlations among traits, which in our case represent genetic differentiation among accessions.

Both a visual inspection of the correlation matrices obtained under either environment and the principal component analyses showed a fairly high degree of similarity between the major aspects of the character architecture as expressed under the two treatments. As indicated by our vector correlation analyses, the first two vectors—which explain over 60% of the observed variance—were highly correlated to each other across treatments. This implies that the environment did not significantly affect the mechanisms underlying character correlations. However, it is also noticeable that many of the minor components were in fact not correlated, if one takes the overall degree of variance explained by each of them as a sufficient matching criterion (which it may not be, especially for the components explaining very small portions of variance).

Lack of matrix divergence among populations has been observed before, for example by Arnold and Phillips (1999) during the work on coastal/inland divergence in garter snakes. Roff and Mousseau (1999) have reviewed the literature on divergence of genetic correlations across different taxonomic levels and found that the results are mixed, as one would expect considering the heterogeneity of methods, types of characters, and taxa that have been employed and sampled so far.

The field of multivariate phenotypic evolution is also plagued by methodological problems. Roff (2000), for example, has compared various methods for examining multivariate genetic/phenotypic divergence and found that no single method yields satisfactory results. Our own experience with the currently popular common principal components (CPC) analyses (Phillips and Arnold, 1999; Waldmann and Andersson, 2000) is actually rather unsatisfying. Both in the case of the current study and in other occasions (Kolodynska and Pigliucci, submt.) we discovered that CPCs tend to be too sensitive and yield a verdict of no similarity among matrices even when it is obvious by both visual inspection and other methods (Mantel tests, vector correlation analyses) that there is in fact a high degree of overlap between matrices. This problem

of finding satisfactory statistical methods to quantify changes in phenotypic integration (see also: Smouse et al., 1986; Cowley and Atchley, 1992; Shaw, 1992) is perhaps the major stumbling block against progress in this important field of inquiry into phenotypic evolution.

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Appendix II.

Table 1. ANOVA table with Mean Squares and associated p-values. Boldface indicates statistically significant effects after a table-wide sequential Bonferroni correction. df indicates the degrees of freedom of each factor. Transformations to compensate for lack of normality or heteroscedasticity are also detailed.

Trait	***Accession (46 df)***	***Treatment (1 df)***	***Accession by Treatment (46 df)***	***Tray (treatment) (22 df)***	***Error (401-435 df)***
Bolting day	**207.34** **0.0000**	11.30 0.3653	20.14 0.0297	**143.86** **0.0000**	13.76
Leaf number at bolting	**84.29** **0.0000**	32.00 0.2588	7.20 0.0364	**23.81** **0.0000**	5.01
Rosette diameter(log)	**0.81** **0.0000**	**37.10** **0.0000**	**0.22** **0.0000**	**0.57** **0.0000**	78.05
Set of first fruits (log)	**0.09** **0.0022**	0.17 0.1910	0.04 0.0336	**0.09** **0.0000**	0.03
Shoot fresh weight (log)	**1.68** **0.0000**	**684.13** **0.0000**	0.49 0.1259	3.31 **0.0000**	0.39
Root fresh weight	0.01 0.0549	**0.18** **0.0000**	**0.01** **0.0000**	**0.01** **0.0000**	0.00
Lateral branches	**4.11** **0.0000**	**309.03** **0.0000**	1.62 0.0733	**2.20** **0.0138**	1.21
Basal stems	4.37 0.0499	**1072.80** **0.0000**	**2.67** **0.0001**	**4.97** **0.0000**	1.30
Total fruit production (log)	**0.85** **0.0000**	**442.20** **0.0000**	0.33 0.0158	**1.48** **0.0000**	0.22

Table 2. Regression analysis conducted on the traits showing plasticity or genetic differentiation for plasticity. The table reports the standardized selection coefficients and the associated t-test and p-values. Boldface indicates statistical significance. Linear and quadratic terms were included in the model. These are normally interpreted respectively as directional and disruptive/stabilizing (depending on the sign) selection.

	Treatment	*Effect*	*Stand. Coeff.*	*t*	*P(2-Tail)*
Linear terms	*Flooded*	Rosette diameter	0.30	6.45	**0.0000**
		Shoot fresh weight	0.35	0.55	**0.0000**
		Root fresh weight	0.10	2.09	0.0372
		N. of lateral branches	0.14	3.86	**0.0001**
		Number of basal stems	0.25	6.21	**0.0000**
	Non-flooded	Rosette diameter	-0.06	-1.15	0.2520
		Shoot fresh weight	0.56	9.89	**0.0000**
		Root fresh weight	0.09	1.60	0.1089
		N. of lateral branches	0.18	5.01	**0.0000**
		Number of basal stems	0.31	8.17	**0.0000**
Quadratic terms	*Flooded*	$(\text{Rosette diameter})^2$	0.25	1.73	0.0855
		$(\text{Shoot fresh weight})^2$	-0.94	-8.86	**0.0000**
		$(\text{Root fresh weight})^2$	-0.17	-2.05	0.0413
		$(\text{N. of lateral branches})^2$	0.10	0.94	0.3493
		$(\text{Number of basal stems})^2$	0.10	1.43	0.1540
	Non-flooded	$(\text{Rosette diameter})^2$	-0.48	-3.26	**0.0013**
		$(\text{Shoot fresh weight})^2$	0.00	-0.02	0.9811
		$(\text{Root fresh weight})^2$	-0.33	-2.89	**0.0042**
		$(\text{N. of lateral branches})^2$	-0.07	-0.55	0.5808
		$(\text{Number of basal stems})^2$	0.03	0.26	0.7939

Table 3. Correlation matrix among characters for flooded conditions. Boldface indicates significant correlations after a Bonferroni correction.

	Bolting day	Leaf No	Root fresh weight	Lateral branches No	Basal stems No	Rosette diameter (log)	Senescence (log)	Shoot fresh weight (log)	Total fruit (log)
Bolting day	1								
Leaf No	**0.77**	1							
Root fresh weight	**0.39**	**0.51**	1						
Lateral branches No	-0.17	-0.04	0.15	1					
Basal stems No	-0.01	-0.04	**0.33**	0.10	1				
Rosette diameter (log)	0.01	**0.34**	**0.47**	0.09	**0.30**	1			
Set of first fruits (log)	**-0.22**	**-0.24**	-0.13	**0.29**	0.18	-0.17	1		
Shoot fresh weight (log)	**0.30**	**0.39**	**0.70**	0.19	**0.44**	**0.72**	-0.14	1	
Total fruit (log)	0.04	0.2	**0.60**	**0.31**	**0.48**	**0.66**	0.05	**0.87**	1

Table 4. Correlation matrix among characters for non-flooded conditions. Boldface indicates significant correlations after a Bonferroni correction.

	Bolting day	Leaf No	Root fresh weight	Lateral branches No	Basal stems No	Rosette diameter (log)	Senescence (log)	Shoot fresh weight (log)	Total fruit (log)
Bolting day	1								
Leaf No	**0.84**	1							
Root fresh weight	**0.56**	**0.59**	1						
Lateral branches No	0.11	0.19	**0.24**	1					
Basal stems No	-0.10	-0.07	**0.23**	0.18	1				
Rosette diameter (log)	**0.66**	**0.68**	**0.71**	**0.38**	**0.33**	1			
Set of first fruits (log)	0.15	-0.01	-0.06	-0.03	0.00	-0.14	1		
Shoot fresh weight (log)	**0.41**	**0.37**	**0.62**	**0.32**	**0.48**	**0.68**	0.04	1	
Total fruit (log)	0.20	0.19	**0.54**	**0.39**	**0.64**	**0.64**	0.07	**0.79**	1

Table 5. Principal Components analyses detailing the composition of the first two eigenvectors under flooded and unflooded conditions. Boldface indicates which component (within each treatment) was associated with the highest load for a given variable.

	Non-flooded		**Flooded**	
Component loadings	**PC-1**	**PC-2**	**PC-1**	**PC-2**
Eigenvalues	*4.13*	*1.76*	*3.66*	*1.95*
Percent of Total Variance Explained	*45.98*	*19.57*	*40.67*	*21.69*
Bolting day	0.43	**-0.72**	**0.69**	0.63
Leaf number	0.58	**-0.65**	**0.70**	0.62
Root fresh weight	**0.83**	-0.09	**0.82**	0.13
Lateral branches	0.20	**0.53**	**0.44**	-0.23
Basal stems number	**0.48**	0.45	0.42	**-0.74**
Rosette diameter	**0.77**	0.11	**0.92**	0.09
Set of first fruits	-0.16	**0.57**	0.00	0.04
Shoot fresh weight	**0.93**	0.12	**0.84**	-0.28
Total fruit	**0.83**	0.40	**0.77**	-0.53

Table 6. Correlations among the principal vectors expressed under the two environmental regimes. Notice how the vectors explaining the majority of the variance appeared to be highly correlated with each other. Several of the smaller vectors, however, did show very low similarity between treatments. Boldface indicates significant correlations after a Bonferroni's correction.

	Vector 1	Vector 2	Vector 3	Vector 4	Vector 5	Vector 6	Vector 7	Vector 8	Vector 9
Pearson's coefficient (flooded vs. non-flooded)	**0.9306**	**-0.8344**	0.4618	-0.6678	-0.0912	-0.3193	-0.1581	**0.9701**	0.5889
(p-value)	(0.0003)	(0.0052)	(0.2108)	(0.0494)	(0.8155)	(0.4023)	(0.6846)	(0.0000)	(0.0953)
Total variance explained for flooded	40.67%	21.7%	11.65%	9.42%	5.94%	4.55%	3.30%	1.92%	0.87%
Total variance explained for non-flooded	45.98%	19.57%	11.88%	9.26%	4.66%	3.66%	2.16%	1.58%	1.25%

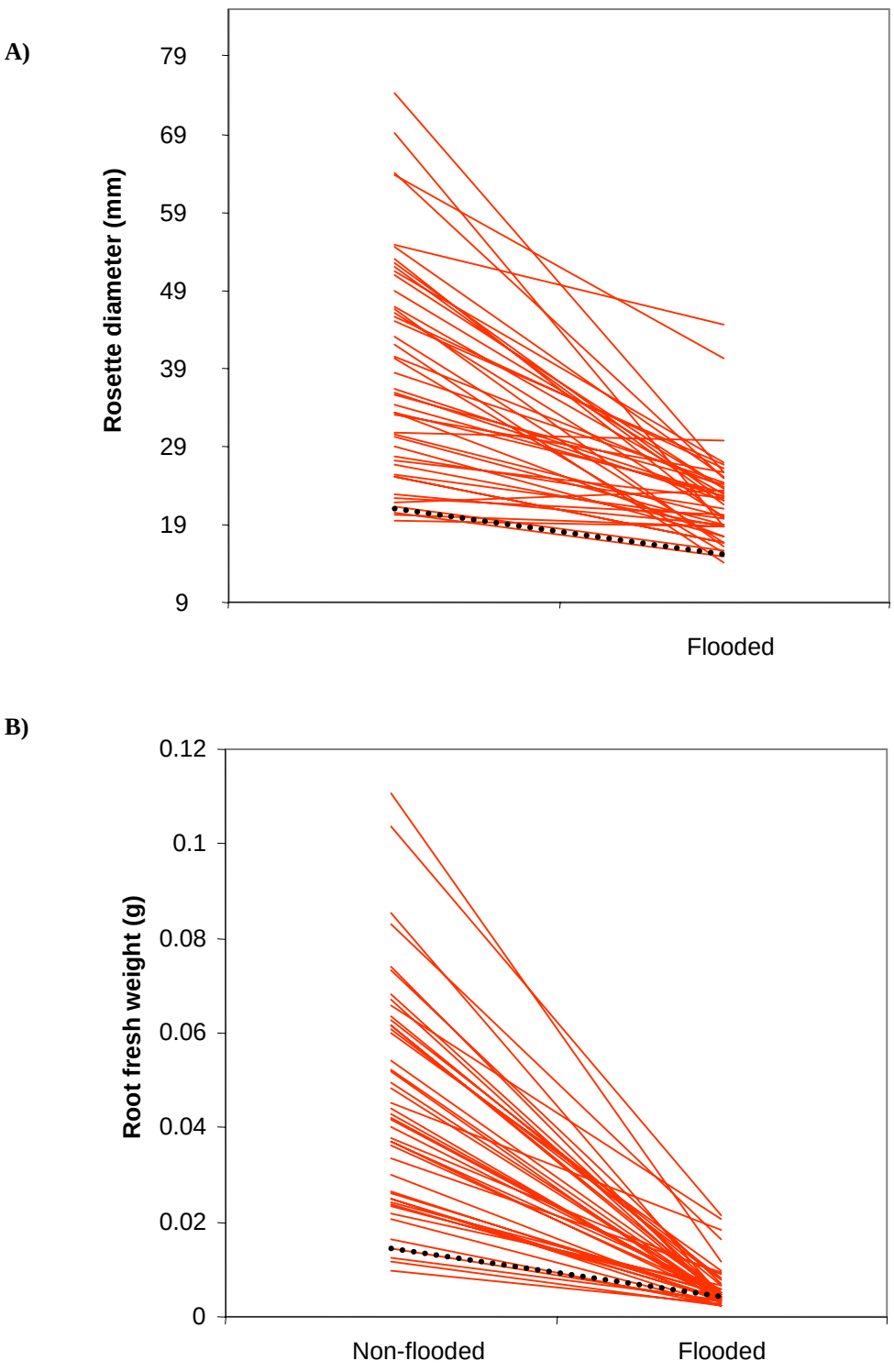


Figure 1. Reaction norms of the traits showing genetic variation for plasticity. The dotted line represents the LER genotype, which is a common laboratory line used in many *Arabidopsis* experiments.

C)

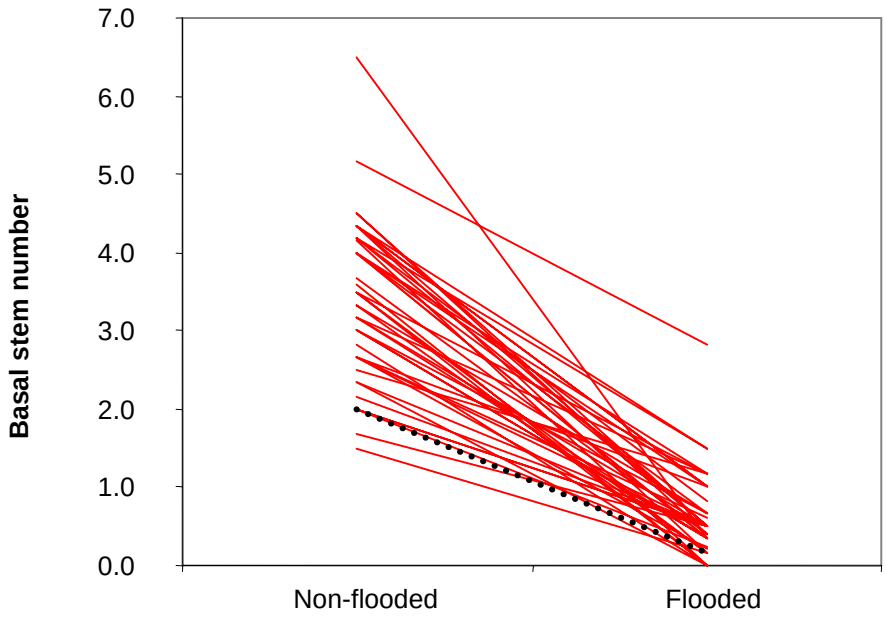


Figure 1. Continued.

A)

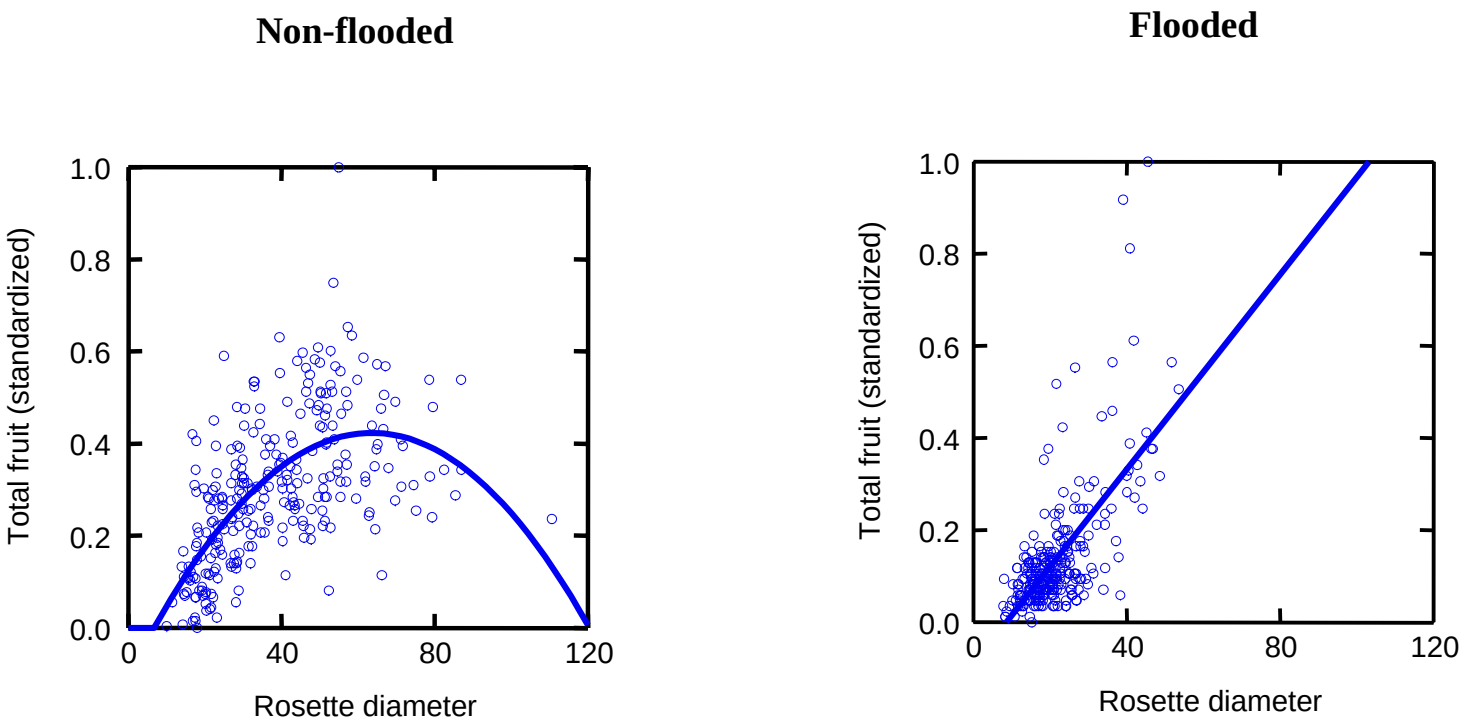


Figure 2. Regression analyses relating the expression of plastic traits and reproductive fitness. The curves represent the best fit to the linear and/or quadratic models.

B)

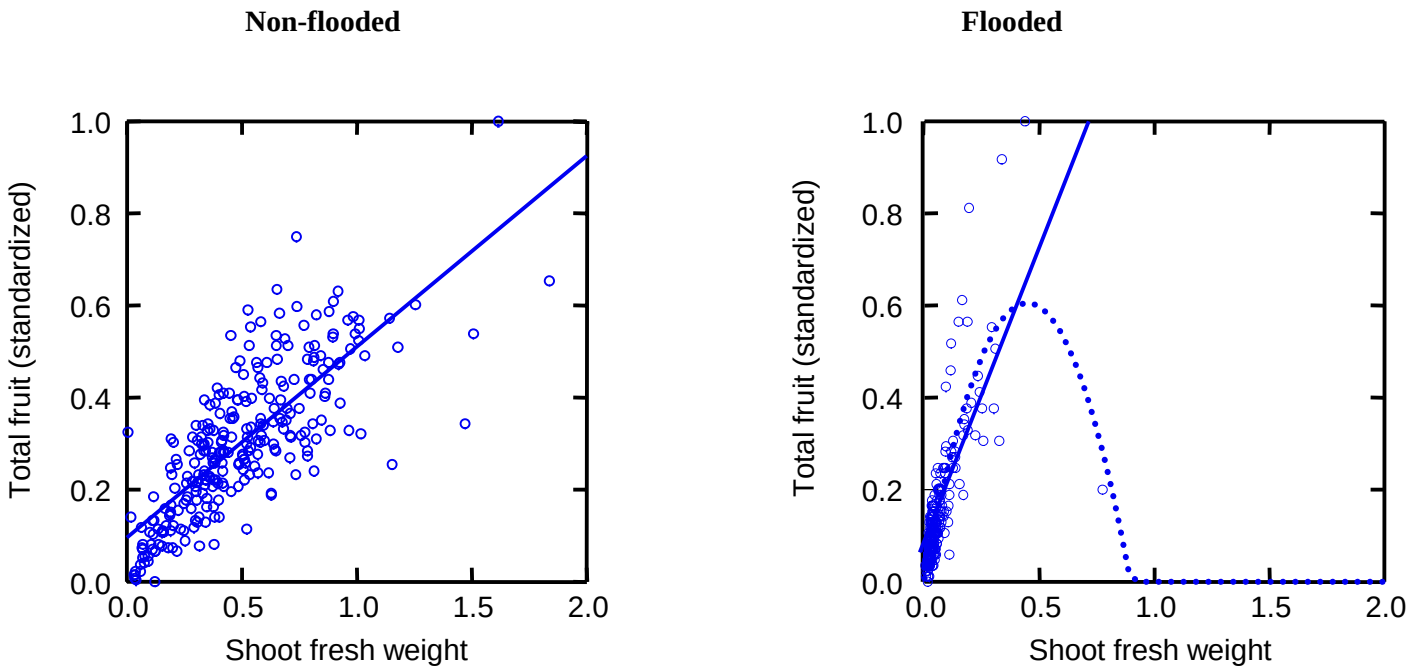


Figure 2. Continued.

C)

Non-flooded

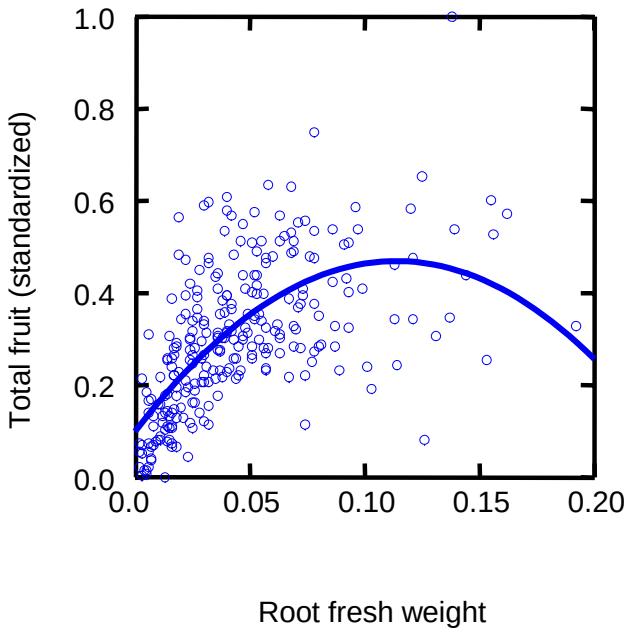


Figure 2. Continued.

D)

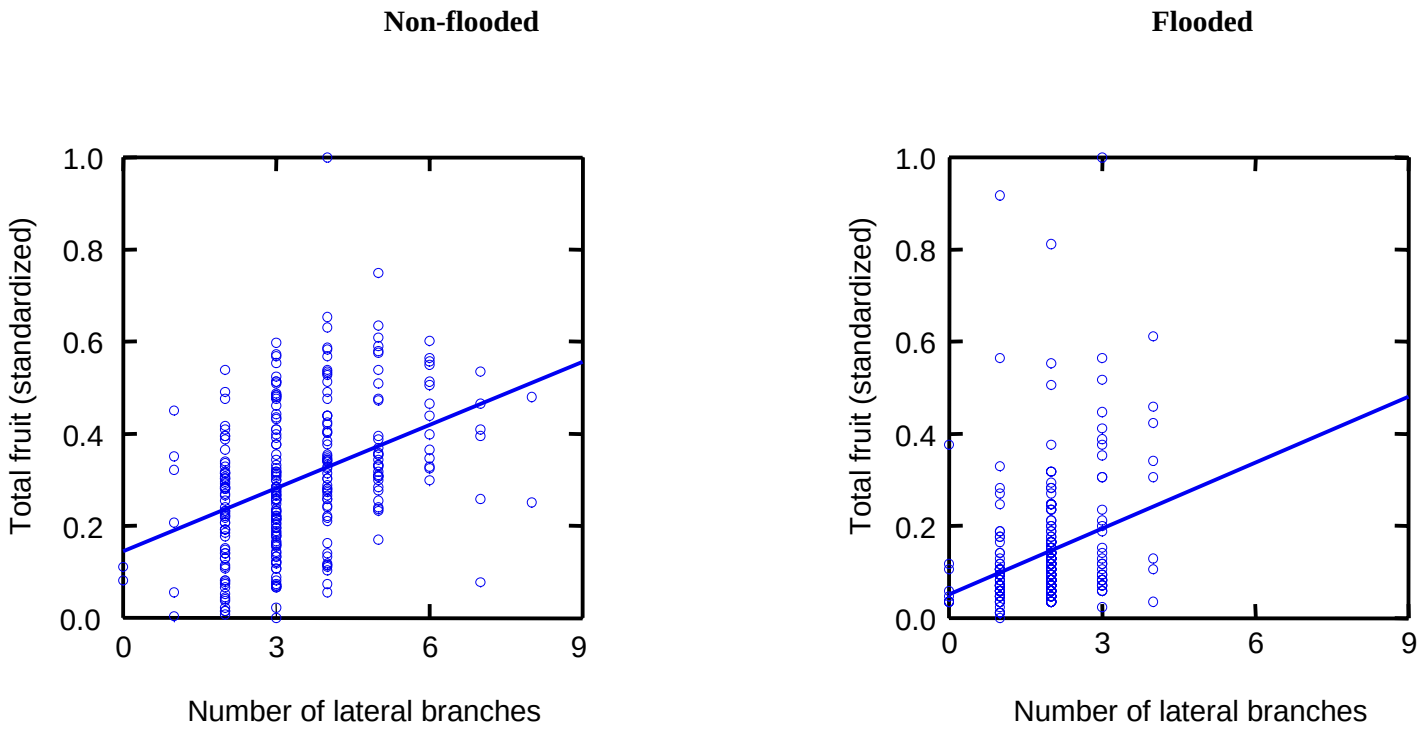


Figure 2. Continued.

E)

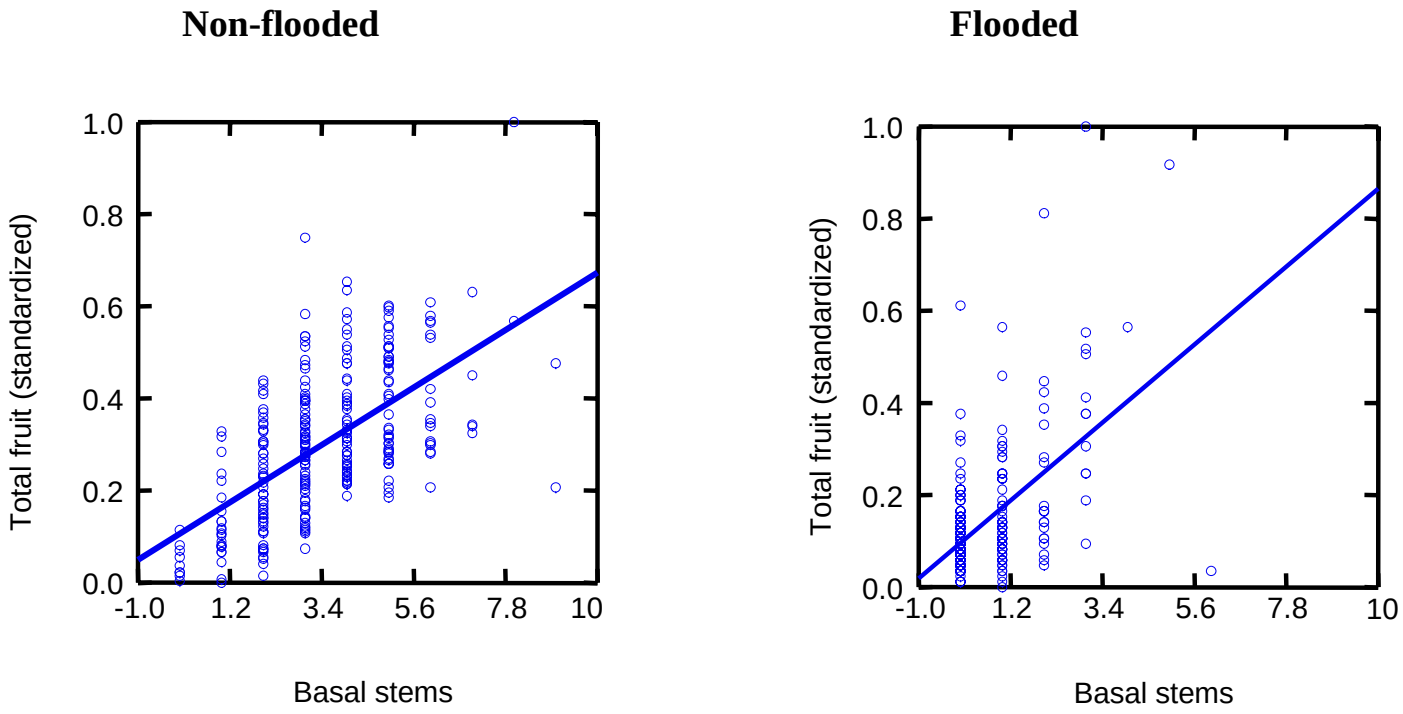
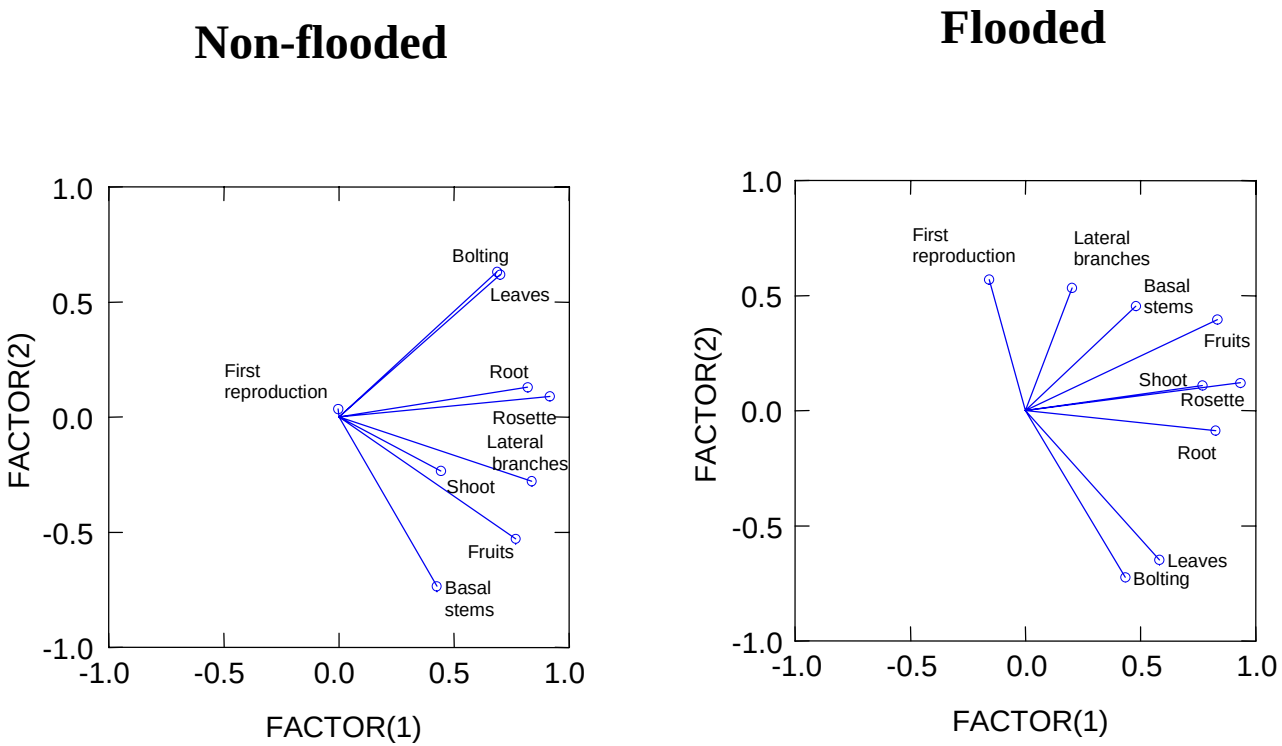


Figure 2. Continued.



Abbreviations used: Bolting = bolting day; Leaves = leaf number at bolting; Rosette = rosette diameter; Roots = root fresh weight; Shoots = shoot fresh weight; Fruits = total fruit production; Basal stems = number of basal stems; First reproduction = days from bolting to first fruit set.

Figure 3. Plot of the Principal Components loadings on the first two eigenvectors. Variables whose vectors are separated by a small angle are highly positively correlated with each other. Diametrically opposed vectors indicate a strong negative association between the corresponding variables.

Part III:

Selection and flood stress in *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae)

Statement:
This part of the dissertation was submitted for publication to the Functional Ecology.

All tables and figures are located in the Appendix III.

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Keywords: *Arabidopsis thaliana*, flooding stress, phenotypic integration, selection, genetic covariances.

Running title: Selection and flooding stress in *Arabidopsis.*

Introduction

Experimental evolution is a promising approach to study the dynamics of natural selection and phenotypic evolution (Bell and Reboud, 1997; Bell, 1997; Ebert, 1998; Becerra et al., 1999). By simulating short-term evolution under controlled conditions it is possible to study both changes in character means and in their variance-covariance matrices, thereby providing insights into the evolution of phenotypic integration (Shiotsugu and Armand M. Leroi, 1997). Lande (1979) and other researchers interested in evolutionary quantitative genetic modeling have assumed that variance-covariance matrices are either invariant or proportional over long periods of time, so that predictions about evolutionary trajectories can be made using relatively simple mathematical models. Bohren et al. (1966) reject such assumption and remark that selection may cause allele frequency changes that substantially and rapidly alter the magnitude and sign of covariances (similar points have been raised, for example, by Turelli 1988; and by Pigliucci and Schlichting 1997). Under this view, long-term evolutionary predictions can be made only by using more elaborate (and likely not analytically solvable) population-genetic models which need to be informed by empirical studies of evolutionary and environmentally-induced changes in character variance-covariance (Bohren and Hill, 1966; Pigliucci and Schlichting, 1997).

Evolutionary changes of both trait means and variance-covariances can be promoted by stressful conditions (Bradshaw and Hardwick, 1989). Stress can be broadly defined as any unfavorable condition of growth; thus, stress is an inevitable—and probably ubiquitous—component of natural environments. Stress can be experienced as a type of fine-grained environmental variation and, as shown by Bell (1997) fine-grained environments may lead to the maintenance of genetic variation, a recurrent problem in evolutionary theory. The grain of the environment can be different on a temporal versus a spatial scale (Bell, 1992), e.g., nutrient availability can be coarse grained on a spatial scale if plants grow on a uniformly nutrient-rich

soil, but it may be fine grained on a temporal scale if availability of nutrients deteriorates as the season progresses.

One of the most important environmental factors for a plant is water availability. In nature, land plants often experience two extreme kinds of water stress: drought or flooding. It is the latter that is the focus of this paper. For plants the primary constrain imposed by flooding is impeded gas-exchange, since diffusive resistance to most gases is approximately 10,000 times greater in water than in air; in addition, there is a 30-fold drop in oxygen concentration between air and water (Armstrong and R. Brandle, 1994). In flooded soils this increased resistance to gas-transport coupled with microbial demand for oxygen means that aerobic conditions can extend from less than 1 mm to no more than a few centimeters from the soil surface. In freshly flooded soils low oxygen concentrations may arise in a matter of hours, and this effect can be accelerated by high temperatures (Armstrong and R. Brandle, 1994), which also catalyze fast replication of microorganisms such as bacteria and fungi, which in turn consume a significant amount of oxygen.

Roots are extremely sensitive to oxygen deficiency and flood-induced stress at the root level is accompanied by depletion of carbohydrates reserves, cytoplasmic pH changes, and ultrastructural changes; as a consequence, cell functionality may be disrupted, nutrient acquisition impaired (Drew, 1997), and hormonal changes induced (Armstrong and R. Brandle, 1994). Indeed, in view of the commonality of at least temporary flooding regimes and their widespread effects on plants, it would be most surprising if tolerance to flooding were based on a single adaptive feature or strategy (Armstrong and R. Brandle, 1994). A key to understand adaptation to flooding therefore resides in a multivariate approach to phenotypic evolution in response to this kind of stress.

The evolution of multivariate patterns of phenotypic covariation is related in important ways to two major concepts commonly employed to understand macroevolution in terms of

microevolutionary processes (Steppan, 1997). First, quantitative genetics models of evolutionary change focus on natural selection acting on the variances and covariances constituting the genetic and phenotypic architecture of a population, which means that understanding changes in variance-covariances is crucial to build such models. Second, evolutionary constraints can play a role in shaping long-term changes in phenotypic covariances insofar the latter are expressions of underlying genetic and/or developmental constraints (Steppan, 1997), which implies that an understanding of constraints requires an understanding of patterns of variation-covariation. As pointed out by several authors (Lande, 1979; Arnold and Wade, 1984; Arnold and Phillips, 1999; Phillips and Arnold, 1999), covariance patterns are fundamental to quantitative genetic models of phenotypic divergence over medium to long periods of time. Thus, if we want to successfully apply microevolutionary models to macroevolution, we need to understand the dynamics of change in character (co)variation and the degree of their temporal stability in the face of evolutionary forces.

In this paper we address these problems by means of a multi-generational study of the effects of selection in response to flooding in the model system *Arabidopsis thaliana* (Griffing and Scholl, 1991; Pyke, 1994; Pigliucci, 1998) and focus on the following questions: 1) How does selection on fruit production (a component of reproductive fitness) under flooding conditions affect genetic variation and reaction norms in a group of natural genotypes of *A. thaliana*? 2) Is high fruit production at the beginning of the selective history a good predictor of the persistence of a given genetic line at the end of the selection process, thereby helping to identify genotypes "pre-adapted" to the conditions under study? 3) How are heritabilities (a standardized measure of genetic variance) affected by contrasting environments and selection regimes? 4) What sort of selection pressures are experienced by individual traits due to their relationship with reproductive fitness? And 5) How do selection and environment affect the

variance-covariance matrix relating different traits, thereby altering the phenotypic architecture in these plants?

Materials and Methods

Bulk collections of seeds from 47 accessions of *Arabidopsis thaliana* (L.) Heynh. were obtained from the *Arabidopsis* Information Management System (AIMS at www.arabidopsis.org): CS0911, Estland (Germany); CS0913, Petergof (Russia); CS0915, Wassilewskija (Russia); CS0916, Condara (Tadjikistan); CS0917, Darmstadt (Czechoslovakia); CS0920, Enkheim (Ukraine); CS0922, Hodja-Obi-Garm (Tadjikistan); CS0925, Litvania (Litvania); CS0932 Aberdeen (UK); CS1184, Gudow (Germany); CS1214, Guckingen (Germany); CS1226, Hilversum (Netherlands); CS1240, Isenburg (Germany); CS1252, Vranov (Czechoslovakia); CS1258, Jamolice (Czechoslovakia); CS1282, Rodenbach (Germany); CS1284, Koeln (Germany); CS1504, Seis (Italy); CS1514, Slavice (Czechoslovakia); CS1604, Wietze (Germany); CS1630, Wildbad (Germany); CS1635, Canterbury (U.K.); CS1637, East Malling (UK); CS1640, Tsu (Japan); CS3109, Copenhagen (Denmark); CS3110, Weiningen (Switzerland); CS3179, Graz (Austria); CS3180, Coimbra (Portugal); CS6003, Koln (Germany); CS6004, Maidstone Kent (U.K); CS6015, West Malling, Kent, (U.K); CS6016, Maidstone (UK); CS6023, Sedmouth (UK); CS6034, Bretagny (France); CS6036, Bretagny (France); CS6038, Kelsterbach (Germany); CS6041, Kelsterbach (Germany); CS6046, Koln (Germany); CS6047, Maidstone, Kent, (U.K); CS6068, Kent (UK); CS6105, Kelsterbach (Germany); CS6187, Washington (USA); CS6194, Blanes (Spain); CS6195, Wurzburg (Germany); and CS6682, Dijon (France). CS6731 Gluckingen, (Germany); and LER, Landsberg erecta.

All accessions selected for our experiment represented early flowering populations of *A. thaliana* and had been bulk propagated at AIMS to maintain genetic variation. In order to

minimize maternal effects and increase seed availability, we grew the material for one generation under controlled laboratory conditions of 16/8 hour of light/darkness at a room temperature of 23-25°C and provided bottom watering every other day to reduce mechanical interference.

These second-generation seeds were placed on a wet filter paper and cold-treated for one week at 5°C in a refrigerator to enhance and synchronize germination. Imbibed seeds were then transferred to a mix of top soil-coarse sand-turface (2-2-1 by volume) and placed under combined fluorescent and incandescent lights on a growth rack. Seedlings were randomly thinned after five days, when the appearance of the first true leaves was noticed, leaving one plant per 7cm diameter by 5cm deep pot. We applied two treatments—non-flooded (control group) and flooded (stress regime). Plants assigned to the non-flooded regime were bottom watered every other day, letting the soil saturate with water for two hours and then drain. The flooded group had water changed every other day (at the time when the control group was watered) to minimize algal growth; pots were never allowed to acquire non-saturated state (i.e., plants were waterlogged at all time during the experiment), with the water level maintained at the top of the soil. Both treatments were top fertilized once a week for 5 weeks with an 11:11:11 (N:P:K) solution in the amount of 2ml to each pot.

Character measurements

At the onset (first generation) and the end (third generation) of the experiment we measured three sets of traits: vegetative, architectural and reproductive. Only fruit production (the selection criterion) was measured during the second generation.

The following *vegetative traits* were measured at the bolting stage, when the rosette begins to produce the flowering stem: 1) Bolting time (time from planting the seeds to the initiation of the main stem); 2) Rosette leaf number, quantifying meristem allocation to vegetative

growth; and 3) Rosette diameter, a measure of plant size during the vegetative phase. *Plant architecture* traits were measured at the time of harvest, one week after maturation of the first fruits (manifested as opening of the siliques on the main inflorescence). At that time we extracted plants from the soil and separated the roots from the shoot by cutting off the latter just below the rosette. The measured architecture traits were: 4) Number of lateral branches; 5) Above ground fresh weight (measuring plant allocation of resources to above ground structures), including the rosette, the main stem, branches, and fruits; 6) Below ground fresh weight (measuring plant allocation of resources to below ground structures); and 7) Total number of basal stems (allocation of resources to secondary meristems), including both elongated and non-elongated basal stems. If a basal stem had opened inflorescences it was counted as an elongated stem, otherwise as a non-elongated one (which measures further *potential* reproductive success). *Reproductive traits* were measured after the plants set the first fruits: 8) Time of first reproduction, when the first seeds matured and the siliques started opening, counted as days from bolting (i.e., from the beginning of the reproductive phase); and 9) Total fruit production (reproductive fitness).

Experimental design and statistical analysis

Plants from each population were randomly assigned to one of the two treatments (flooded or non-flooded), with every population represented by six replicates within each treatment. The total size of the experimental population was therefore 47 families by 2 treatments by 6 replicates = 564 plants. Individuals were placed in two growth racks with each rack housing three shelves, with four trays on each shelf. Each shelf contained two replicates of each family randomly assigned to one of the four trays, yielding 94 individual plants per shelf and 23-24 pots per tray.

For each treatment we established two replicated selection lines, yielding a total of four lines: flooded one, flooded two, non-flooded one and non-flooded two. Members of each line were grown on the same shelf, individual plants being randomly arranged on each shelf. Plants were grown following this setup for three generations. At the end of each generation the top 33% individual plants (regardless of their accession of origin), from each selection line were chosen based on fruit production. Seeds from these plants were used to establish the next generation and continue the experiment. Thus, each selected individual plant yielded three progeny in such a way that while we were possibly loosing some of the initial accessions, the total size of the experimental population was preserved. Notice that this procedure yields largely selection by line sorting, since these plants are highly selfing (Abbott and Gomes, 1989), although occasional outcrossing may have occurred by mechanical cross-stimulation of adjacent individuals. This is the way natural selection likely works on a plant like *Arabidopsis* under field conditions, so we consider the set up to be a rather reasonable approximation to realistic population dynamics in this respect.

Measured variables deviating from normality or homoscedasticity were appropriately transformed (Sokal and Rohlf, 1995). We employed a nested mixed-model analysis of variance (split-plot design: SYSTAT, 2000) to estimate the significance of the following factors: Generation, testing the overall effect of change in character means due to selection. Treatment, estimating overall phenotypic plasticity independent of selection, genetic accession, etc. Line (nested within Treatment), testing for differences between selection lines due, for example, to drift. Shelf, estimating the degree of micro-environmental variation due to the experimental setup. Generation × Treatment, testing for differences in the response to selection depending on the environment experienced. Generation × Line (within Treatment), testing for differences between selection lines during the course of selection. Generation, Line and Treatment were considered fixed effects, while Shelf was treated as a random effect. According to Sokal and Rohlf (1995), if

the Line(Treatment) effect is significant, then the Treatment effect is to be tested over Line(Treatment). Also, if the Generation × Treatment interaction shows a significant effect, it is more conservative to test Generation and Treatment over the interaction term. Otherwise, factors were tested over the error mean square (this judicious use of conservative statistical tests is advocated by Sokal and Rohlf, and we consider it better than always testing over interactions or lower-level effects, even when these are not significant). Given the high number of multiple comparisons (nine traits), we used a sequential Bonferroni correction to adjust the nominal α-values for the ANOVAs across rows in Table 1 (again, this correction is moderately conservative, as opposed to a straight Bonferroni, which tends to overcorrect for type II errors: Rice, 1989).

We conducted a rank test of the estimate of reproductive fitness (fruit production), comparing the ranks of the same accession at the beginning and at the end of the selection process to see if its initial fitness was a good predictor of the persistence of a given accession from the first through the last generation. We also used the H^2Boot program (provided by Patrick Phillips, at http://www.uoregon.edu/~pphil/) to calculate heritabilities for traits measured at the beginning and end of the selection process.

We performed an additional analysis of variance on the subset of accessions that had a representative in both treatments in the F_3 generation. The 21 accessions thus included were subjected to the same analysis of variance already described, with the addition of the following terms: Accession, which provides information on genetic differentiation among plants from different provenances, regardless of other effects. Accession × Generation, which estimates variation in individual accessions' response to selection; Accession × Treatment, to test whether there was genetic variation for plasticity regardless of other factors; Accession × Line(Treatment), to test whether accessions behaved differently if assigned to different replicated selection lines; and Accession × Generation × Treatment, a test of the ability of selection to alter

genetic variation for plasticity. We used the three-way interaction term (Generation × Treatment × Accession), when statistically significant, as an error term for testing the significance of the main effects (otherwise, they were tested over the general error term, as usual).

We carried out calculations of heritabilities on both the full and the reduced sets of accessions. Using the full set, we also plotted reaction norm diagrams and conducted regression analyses of each plastic trait against reproductive fitness, both before and after selection. The regression analyses aimed at obtaining information on the type of selection (directional or stabilizing) that was operating on the traits that were responding to the environmental conditions.

In order to test whether the phenotypic architecture (measured by correlations matrices and multivariate statistics) had changed after selection, we performed principal components analyses and vector correlation analyses (on the full set of accessions), comparing factor loadings from the first and last generations of the experiment.

Results

Reaction norms and variation within and across generations

The analysis of variance on the full data set revealed that selection (Generation effect) had an effect on all of traits except root weight (Table 1). There was overall plasticity (Treatment effect) for all of traits except bolting time. There were no statistically significant differences between selection lines since none of the traits was significant for the Line nested within Treatment effect. Most traits showed statistically significant micro-environmental effects (Shelf factor), with the exception of rosette diameter, root weight and number of lateral branches. The effect of selection varied with the environment (generation by treatment interaction) for reproductive (except the timing of first reproduction) and architecture traits, but not for the

vegetative traits. Selection lines did not respond differently to the imposed selection regime (non-significant Generation by Line within Treatment effect).

The analysis of variance performed on the subset of the accessions that survived the selection process (Table 2) showed that selection had a widespread effect on all of the traits measured (Generation effect). We also observed widespread plasticity (six out of nine traits were statistically significant for the Treatment effect, the exceptions being bolting time, leaf number and time of first seed set). There was widespread genetic variation among accessions, with all traits showing significant differences. Selection lines did not differ in their overall behavior (no significant Line within Treatment effect). Rosette diameter and number of lateral branches were the only traits not showing a micro-environmental (Shelf) effect. As in the full data set, the effects of selection differed between treatments for all architecture and reproductive traits except timing of first reproduction (Generation by Treatment effect), but not for the vegetative characters. The individual accession's response to selection was different for vegetative traits such as bolting time, leaf number at bolting, and rosette diameter and rather uniform for the remaining ones (Accession by Generation effect). We detected genetic variation for plasticity across generations in traits such as rosette diameter, time of first reproduction, and root fresh weight (Accession by Treatment effect). Leaf number at bolting and rosette diameter were the only two traits that differed in their response to selection depending on the replicated lines (significant Generation by Line within Treatment effect). Accessions did not behave differently when attributed to distinct selection lines (none of the traits was significant for the Accession by Line within Treatment effect). Also, selection did not alter genetic variation for plasticity for any of the measured traits, since none of the three-way interaction terms was statistically significant.

A visual inspection of the data showed that the reaction norms of the plastic traits conserved the general pattern of plasticity between generations (Fig. 1A-H): plants consistently produced higher phenotypic values under non-flooded conditions than under flooded ones for

almost every trait (with the exception of those with no significant overall treatment effects: bolting time, leaf number and time of first seed set). However, we did observe changes in the expressed variance and overall mean of some of the traits in response to selection. For example, root fresh weight (Figure 1 E) dropped dramatically from the beginning to the end of the selection period, and so did the number of lateral branches (Fig. 1 F) and the number of basal stems (Fig. 1 G).

Heritabilities

We observed a change in the heritabilities of some traits both within treatments and between generations (Table 3). In the first generation, traits such as time of set of the first fruits, number of lateral branches, and number of basal stems did not have statistically significant heritabilities under the flooded treatment, but their heritabilities were significant under non-flooded conditions. In the third generation, on the other hand, traits such as shoot weight and number of basal stems had statistically significant heritabilities under flooded conditions but not under non-flooded ones. In addition, a significant heritability of fruit production (regardless of treatment) observed in the first generation became not significant in the third generation, confirming the efficiency of the selection process in reducing genetic variation for fitness.

We performed these calculations also for the subset of lines present across the entire selection experiment (Table 4). As it may be expected, these showed less variation between treatments and generations, and the variation we did observe was presumably due to within-line genetic variance rather than to the process of line sorting. The main changes concerned the timing of set of first fruits, which became statistically significant under non-flooded conditions in the third generation (although it had the same magnitude under flooded conditions in the same generation), and root fresh weight, which had a statistically significant heritability in the first generation under flooded conditions but not under either treatment in the third generation.

Compared to Table 3, the heritability of number of basal stems became significant in the third generation / unflooded conditions and the heritability of fruit production was never significant, even in the first generation (again, confirming that the response to selection was due to line sorting).

A rank test performed on reproductive fitness (fruit production) of the full data set showed that high fruit production in the first generation was indeed a good predictor of survival of a given accession from generation one to generation three (variation coefficient = 53.60, p-value <0.0001), again suggesting line sorting as the main operant process.

Relationship between traits and reproductive fitness

Regression analyses performed on the traits showing plasticity (significant Treatment effect or interactions including treatment in Table 1) and their relationship with reproductive fitness showed that leaf number at bolting was under directional selection for a decrease in the trait value during both generations and under both treatments (Tables 5 and 6). In the third generation, under flooded conditions there was also significant stabilizing selection for number of leaves at bolting time (Table 6).

Rosette diameter showed a significant linear effect on fitness under both treatments in the first generation, and under the non-flooded environment in the third generation. In the first generation, rosette diameter showed also a significant quadratic component indicating stabilizing selection under non-flooded conditions and apparent disruptive selection under flooded conditions; however, after visual inspection the latter turned out to be a case of non-linear directional selection (details not shown).

Timing of the first reproduction was under linear selection during the first generation under both treatments; in addition, we detected stabilizing selection under non-flooded conditions

in the first generation, though the latter seemed weakly supported after a visual inspection of the data (not shown).

Shoot weight was under directional selection in all combinations of treatment and generation. We detected significant apparent disruptive selection on this character under non-flooded conditions during both the first and third generations, but again a visual inspection (details not shown) revealed that this was due to a few outliers and that a linear effect was all that was needed to explain the observed variance.

A linear effect on fitness was statistically significant for root weight only during the first generation under non-flooded conditions. No quadratic effects were significant for this character.

Directional selection for increase in number of lateral branches was detected under both treatments and generations (first and last). Also in this case no quadratic effects were observed.

Similarly, there was directional selection for an increase of the number of basal stems in all combinations of treatments and generations. There was stabilizing selection on this trait under non-flooded conditions in both generations, while apparent disruptive selection under flood in the first generation turned out to be due to a quirk of the regression fit algorithm and the linear term was clearly sufficient to explain the covariance between this character and reproductive fitness (details not shown).

Phenotypic integration

In order to explore whether selection changed the structure of phenotypic correlations in our population we performed a series of principal components analyses accompanied by vector correlation analyses. In the first generation, under non-flooded conditions most (all but number of basal stems and set of first fruits) factor loadings were associated with the first principal component (explaining 46% of the variance), while basal stems number weighed heavily on the

second vector (Fig. 2a). Also in the first generation, but under flooded conditions, most of the high loadings were again on the first vector, which explains 41% of the total variance; the exceptions were bolting time, leaf number and lateral branching, which were more strongly associated with the second principal component (Fig. 2b).

At the third generation, the multivariate architecture looked different from the beginning of the selection process, with a broader spread of the traits and a more pronounced effect of the timing of fruit set on the first two principal components (Fig. 2c,d). At the same time, the relevance of root production under flood had visibly diminished when compared to both the non-flooded F_3 and either treatment in the F_1 generation (Fig. 2d).

Vector correlation analyses performed on all possible combinations of generations and treatments showed that the first two vectors, explaining over 60% of the total variance, were generally highly correlated in the first generation (Table 7) but that the correlation became less significant for the first vectors expressed in the third generation (Table 8). Other minor shifts were observed when some of the less important eigenvectors (those explaining less than 5% of the total variance) were compared.

We also observed a high degree of concordance of the major eigenvectors between the first and third generation under non flooded conditions (Table 9), but less so under flooded conditions (Table 10). Again, minor variations within these contrasts were observed for some of the smaller eigenvectors.

Discussion

Experimental evolution is a powerful approach to determine how selection molds an organism's life history and phenotype and can yield insights into the evolutionary impact of particular environmental factors on the genetic structure of an evolving population. This is

especially relevant when evolution occurs under conditions of stress, which may be the most common situation encountered by plants in nature (Stanton et al., 2000).

In this study an artificial population of *Arabidopsis thaliana* was subjected to soft selection that resulted in a dramatic change of the population's genetic make-up. Selection under both flooded and non-flooded conditions caused several accessions to go extinct, altering the available genetic variance (heritability) of several characters as well as the covariances among traits. Since line sorting is presumably the chief selection regime operating in *A. thaliana* under field conditions (because of its high degree of selfing: Abbott and Gomes, 1989), our results imply that even populations characterized by a large initial genetic variation may rapidly converge on a specialist-like phenotypic syndrome (Schlichting and Pigliucci, 1998, chapter 9). In the following, we address the questions we outlined in the Introduction in the light of the results of our selection experiment.

How does selection on fruit production under flooding conditions affect genetic variation and reaction norms?

Selection significantly affected all of the measured traits but did not change the overall shape of the reaction norms and therefore alter the degree of phenotypic plasticity. Not surprisingly, genetic variation decreased from the level in the base population to the third generation, since several accessions went extinct during the process of line sorting under both environmental conditions (we observed a loss of 13 accessions under flooded conditions and of 18 accessions under non-flooded). A similar result was found by Bell (1997) during his work on the unicellular chlorophyte *Chlamydomonas*: a population grown under uniform environmental conditions (like our selection lines) showed much more severely reduced genetic variance for fitness than when the experiment was conducted under heterogeneous environmental conditions.

In our experiment we further observed that selection for increased reproductive fitness moved the population toward similar character means regardless of the environmental conditions (most trait means were lower under flooded than non-flooded conditions by the end of the experiment, but this was also true at the beginning). This has several implications: first, apparently selection on reproductive fitness could be effected by similar shifts in character means regardless of the environment. Second, this shift was similar to the natural genotypic response induced by the stress environment, implying that selection for increased reproductive fitness was equivalent to imposing a stressful condition. Third, the evolutionary response of our plants was characterized by a mostly parallel shift in the reaction norms of the population: the degree of plasticity did not change, while the across environment mean went down significantly for several traits.

Few experiments have been conducted on the effects of artificial selection on the shape of reaction norms. Scheiner and Lyman (1991), for example, selected in two different fashions on reaction norms of *Drosophila melanogaster* to temperature, and did obtain a change in both plasticity and character means. The difference between our experiment and theirs, of course, is that we did not select on plasticity per se; in fact, our population experienced only one environment during the experiment (either flooded or non-flooded), with the plasticity being gauged at the beginning and at the end of the selection process. More research on the evolutionary flexibility of reaction norms is obviously needed, especially in light of reports of strong genetic constraints that may be acting differentially on the shape and height of the reaction norms of certain traits (Pigliucci et al., 1998).

Is high fruit production at the beginning of the selective history a good predictor of the persistence of a given genetic line at the end of the selection process?

As we expected based on the idea that selection would proceed by line sorting (despite the possibility of occasional outcrossing due to mechanical contact among adjacent plants), accessions with high fruit production in the first generation were indeed more likely to find themselves among the survivors at the end of the selection process. It would be interesting to relate this success to the sort of environments that these accessions experienced in the field before collection, but this information is currently rarely available for *Arabidopsis,* although efforts are currently in place to better understand its life history and autoecology (Callahan and Pigliucci, in press).

In general, however, our results are consistent with the idea that our selection regimes were not unusual for these plants, since the high predictability of the "winners" at the end of the experiment shows that some lines were "pre-adapted" to the selective pressures we applied during the experiment. If our environments had been completely novel, no particular relationship would have been expected between the reproductive fitness of the accessions at the beginning and at the end of the experiment.

Much research has been carried out on the effects of exposure of organisms to novel environments (e.g., Service and Rose, 1985; Holloway et al., 1990; Joshi and Thompson, 1996; Hawthorne, 1997; Callaway and Aschehoug, 2000) as well as on the related problem of the relationship between natural and laboratory conditions and how the latter are informative on the former (Matos et al., 2000; Sgro` and Partridge, 2000; Hoffmann et al., 2001). The major stumbling block for further progress here is that there rarely such thing as a truly "novel" environment, so that the debate has misleadingly been cast so far in an essentialist frame which contrasts natural vs. artificial, or old natural vs. novel natural. The truth is that environments are

likely perceived by organisms as relatively more or less novel depending on the correlations that the "new" environmental features share with those of standard or older environments. Unfortunately, it is a time consuming and logistically non-trivial task to estimate the degrees of such correlations.

How are heritabilities affected by contrasting environments and selection regimes?

We have detected strong heritabilities for traits such as bolting time, leaf number at bolting, rosette diameter, and root weight that remained significant across generations and treatments. We observed lower degrees of heritability for life history traits such as set of first fruits and total fruit production when compared to morphological characters like leaf number or rosette diameter. Strong heritabilities for morphological traits and weaker for life-history traits were reported in a review of the literature by Weigensberg and Roff (1996), who analyzed field estimates of narrow sense heritabilities derived from the literature and compared them with estimates from laboratory studies on wild, out-breeding animal populations.

We calculated heritabilities on two sets of data, first using all accessions that were present at the beginning of the selection experiment and then using only those accessions that had persisted under both treatments at the end of the selection protocol. Comparison of these two data sets showed that strong heritabilities remained so even when the genetic variation in the population decreased due to loss of accessions, one exception being root weight, the heritability of which became not significant in the subset of the accessions. A similar decrease in heritability was observed for number of lateral branches, which also showed no significant heritability in the final subset of accessions despite initially moderate levels of heritability in the full data set.

It is now widely recognized that heritabilities can be affected not only by the degree of genetic variation in a population, but also by the sort of environment in which they are calculated

(a fact first noticed by Lewontin, 1974). For example, Ryan (2001) studied morphological traits in a hybrid buntings complex (*Nesospiza*) and estimated heritabilities from parent-offspring and sib-sib regressions. He attributed the difference between the two to differences in the environmental factors experienced by the two generations. More direct demonstrations of influences of biotic factors such as competition and density on heritabilities are found in studies on alfalfa by Asay et al. (1999) and by Mazer and Schick (1991) in *Raphanus*. In general, it is no longer possible to consider heritabilities as anything more than highly local measures of genetic variation for quantitative traits which are bound to be dramatically altered by both the genetic constitution of a population and the environments it experiences.

What selective pressures are experienced by individual traits under different environmental conditions?

We observed strong phenotypic plasticity for almost all examined traits, and the reaction norms were such to indicate that the flooded conditions were indeed a major stress for these plants: most character means were much lower under the flooded than the non-flooded treatment, resulting in plants with smaller rosettes, shorter shoots, smaller roots, fewer branches (both lateral and basal) and reduced fruit production. Interestingly, however, the stress condition did not seem to affect meristem allocation to leaves, and had little or no effect on the two life history characters of length of the vegetative and reproductive phases. Our regression analyses aimed at exploring the relationship between plastic traits and reproductive fitness showed that all plastic traits were under directional selection in at least one combination of treatment/generation, and more so at the beginning than at the end of the selective history.

Interestingly, most traits were selected for an increased in their values (except for bolting time, with plants bolting earlier being characterized by higher reproductive fitness). This is in contradiction to the observation that the reaction norms of some traits shifted downwards between the beginning and the end of the selection experiment and none shifted upwards (see above). The simplest explanation of these opposite patterns is to be found in the observation that our selection protocol actually failed to increase reproductive fitness: the reaction norms for that trait were about the same at the beginning and at the end of the experiment. This in turn may partly be the result of the fact that selection occurred mostly by line sorting: we managed to reduce genetic variance for a variety of traits (including reproductive fitness), but could only shift their mean value within the initial range of variation present in our population. If line sorting is typical of natural populations of *A. thaliana* in the field, then selection is expected to proceed by occasional bursts made possible either by the appearance of new mutants (Pigliucci et al., 1998) or by rare outcrossing events (Abbott and Gomes, 1989).

We found little if any evidence of non-linear selection (disruptive or stabilizing) on the traits we considered, with most cases of significant quadratic coefficients turning out to be due to a few outliers of to slightly non-linear cases of directional selection. One of the interesting exceptions was rosette diameter, which in our F_1 was under stabilizing selection in the non-flooded conditions and under non-linear directional selection in the flooded treatment. This can be explained by the combination of two factors: on the one hand there seem to exist an actual concave function favoring intermediate leaf size in *A. thaliana*, presumably because rosettes that are too little do not generate enough photosynthate while too large ones are metabolically costly. On the other hand, our plants exhibited a limited phenotypic range under flood because of the stress they were under, and the corresponding regression analysis picked only the left portion of the concave fitness function. Interestingly, an almost identical situation—presumably reflecting

similar underlying factors—was observed for the production of basal stems under the two environmental regimes.

While one could object that studying selection under artificial conditions is not particularly informative, we suggest that on the contrary this may yield more clear results than studies conducted in natural environments simply because the latter are confounded by a large number of factors that tend to cancel each other and yield highly variable and weak estimates of selection coefficients (Hoekstra et al., 2001; Kingsolver et al., 2001). Of course, for studies under controlled conditions to be ecologically informative they have to be carried out under a simplified set of realistic conditions, which represents a challenge in the case of the many species for which we don't have an accurate characterization of the autoecology (see above).

How do selection and environment affect the variance-covariance matrix relating different traits, thereby altering the phenotypic architecture in these plants?

While classical studies in evolutionary ecology tended to focus on variation in single characters (with some notable exceptions: Berg, 1960; Clausen and Hiesey, 1960), there has been a recent increase in interest in the co-variation among traits and its consequences for phenotypic evolution (Steppan, 1997; Arnold and Phillips, 1999; Phillips and Arnold, 1999; Waldman and Anderson, 2000). We were particularly interested in the relationship between phenotypic integration (assessed by the pattern of character correlations) and environmental variation, i.e., in how the environment can alter the patterns of phenotypic correlations among traits, which in our case represent genetic differentiation among accessions.

Both a visual inspection of the correlation matrices obtained under either environment and the principal components analyses showed a fairly high degree of similarity between the major aspects of the character architecture as expressed under the two treatments in the first

generation of the experiment. As indicated by our vector correlation analyses, the first two vectors—which explained over 60% of the observed variance—were highly correlated to each other across treatments in the initial generation. This implies that the environment did not significantly affect the mechanisms underlying character correlations. However, the picture was different in the third generation, where only the second principal components were correlated (other than some of the minor eigenvectors, which explained less than 5% of the total variance). We therefore conclude that while environmental variation per se did not affect the patterns of phenotypic integration, the selection process did as a result of the elimination of some accessions and of the increased representation of some lines in the final population.

Several authors have observed a certain degree of conservativeness of phenotypic integration matrices among populations, for example in the case of a study by Arnold and Phillips (1999) on coastal/inland divergence in garter snakes. Roff and Mousseau (1999) have reviewed the literature on divergence of genetic correlations across different taxonomic levels and found that the results are mixed, as one would expect considering the heterogeneity of methods, types of characters, and taxa that have been employed and sampled so far. Future work will have to address the obvious problem that phenotypic or genetic "architectures" can be a highly heterogeneous category depending on the specific traits and organisms being considered.

The field of multivariate phenotypic evolution is also plagued by methodological problems. Roff (2000), for example, has compared various methods for examining multivariate genetic/phenotypic divergence and found that no single approach yields satisfactory results. Our own experience with the currently popular common principal components (CPC) analyses (Phillips and Arnold, 1999; Waldmann and Andersson, 2000) is actually rather unsatisfying. Both in the case of the current study (not shown) and in other occasions (Pigliucci and Kolodynska, in press) we discovered that CPCs tend to be too sensitive and yield a verdict of no similarity among matrices even when it is obvious by both visual inspection and other methods (Mantel tests,

vector correlation analyses) that there is in fact a high degree of overlap between matrices. This problem of finding satisfactory statistical methods to quantify changes in phenotypic integration (see also: Smouse et al., 1986; Cowley and Atchley, 1992; Shaw, 1992) is perhaps the major stumbling block against progress in this important field of inquiry into phenotypic evolution.

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Appendix III

Table 1. Analysis of variance (means square) of the full data set, which included all accessions present at the end of the selection experiment. Degrees of freedom for each effect are given in parenthesis at the heading of each column, significant p-values were adjusted by a sequential Bonferroni correction.

Trait	Generation (G) (1 df)	Treatment (T) (1 df)	Line(Treat) (L(T)) (2 df)	Shelf (2 df)	G×T (1 df)	G×L(T) (2 df)	Error (1051-1102 df)
Bolting time	**6662.51** **0.0000**	142.04 0.0329	47.05 0.2211	**404.51** **0.0000**	65.90 0.1460	23.77 0.4662	31.13
Leaf number at bolting	**209.14** **0.0000**	**168.71** **0.0001**	4.23 0.6828	**85.62** **0.0005**	38.36 0.0630	2.98 0.7643	11.07
Rosette diam (log)	**2.49** **0.0006**	**86.10** **0.0000**	0.30 0.2440	0.96 0.0102	0.32 0.2191	0.50 0.0908	0.21
Set of first fruits (log)	**3.13** **0.0000**	**0.75** **0.0000**	0.11 0.0613	**0.34** **0.0002**	0.27 0.0097	0.003 0.9227	0.04
Shoot fresh weight (log)	**12.62** **0.0000**	**994.11** **0.0000**	1.75 0.0745	**15.77** **0.0000**	**32.19** **0.0000**	0.10 0.8564	0.67
Root fresh weight	0.01 0.1016	**0.19** **0.0000**	0.003 0.4634	0.002 0.6383	**0.04** **0.0017**	0.005 0.3072	0.004
Lateral branches No	**111.66** **0.0000**	**354.71** **0.0000**	0.48 0.6899	3.95 0.0479	**37.11** **0.0000**	0.10 0.9266	1.29
Basal stems	**93.03** **0.0000**	**1239.79** **0.0000**	0.93 0.5337	**27.02** **0.0000**	**147.02** **0.0000**	0.23 0.8580	1.47
Total fruit production (log)	**4.95** **0.0005**	**655.44** **0.0000**	0.55 0.2643	**6.56** **0.0000**	**18.83** **0.0000**	0.03 0.9366	0.41

Table 2. Analysis of variance (means square) performed on a subset of 21 accessions, those present in both treatments in the final generation of the selection experiment. Boldface marks significant effects after a table-wise sequential Bonferroni correction.

Trait	Generation (G) (2 d.f.)	Treatment (T) (2 d.f.)	Accession (A) (20 d.f.)	Line(Treatment) (L(T)) (2 d.f.)	Shelf (5 d.f.)	G×T (1 d.f.)	A×G (20 d.f.)	A×T (20 d.f.)	G×L(T)	A×L(T) (40 d.f.)	A×G×T (20 d.f.)	Error (635 d.f.)
Bolting time	**3007.49** **0.0000**	51.90 0.0481	**239.96** **0.0000**	27.41 0.1269	**138.47** **0.0000**	16.96 0.2581	**62.66** **0.0000**	9.92 0.7749	27.08 0.1302	19.29 0.0370	21.43 0.0434	13.23
Leaf number at bolting	**178.40** **0.0000**	26.41 0.0131	**94.74** **0.0000**	16.86 0.0198	**32.74** **0.0005**	7.76 0.1781	**34.84** **0.0000**	4.63 0.3598	**24.26** **0.0036**	4.54 0.3683	5.24 0.2246	4.27
Rosette Diameter (log)	**9.14** **0.0000**	**21.57** **0.0000**	**1.93** **0.0000**	0.32 0.0780	0.39 0.0439	0.0008 0.9346	**0.33** **0.0001**	**0.31** **0.0003**	**0.84** **0.0012**	0.17 0.0541	0.17 0.1274	0.12
Set of first fruits (log)	**0.92** **0.0000**	0.16 0.0379	**0.19** **0.0000**	0.005 0.8744	**0.30** **0.0003**	0.14 0.0487	0.07 0.0153	**0.09** **0.0004**	0.02 0.6553	0.06 0.0157	0.04 0.5042	0.04
Number of lateral branches	**69.15** **0.0000**	**93.23** **0.0000**	**5.11** **0.0000**	0.28 0.7791	2.26 0.1341	**21.04** **0.0000**	1.57 0.1137	1.95 0.0243	0.88 0.4553	1.87 0.0071	1.08 0.5031	1.12
Shoot weight (log)	**34.07** **0.0000**	**334.28** **0.0000**	**2.73** **0.0000**	0.13 0.7857	**10.00** **0.0000**	**8.19** **0.0001**	0.64 0.2744	0.74 0.1378	1.51 0.0651	0.70 0.1248	0.80 0.0899	0.55
Root weight	**0.0250** **0.0000**	**0.0796** **0.0000**	**0.0017** **0.0000**	0.0003 0.4317	**0.0030** **0.0003**	**0.0147** **0.0000**	0.0006 0.0450	**0.0009** **0.0004**	0.0004 0.3542	0.0004 0.3792	0.0005 0.1523	0.0004
Number of basal stems	**80.13** **0.0000**	**430.86** **0.0000**	**6.85** **0.0000**	0.35 0.7474	**13.10** **0.0000**	**67.07** **0.0000**	1.71 0.1022	2.21 0.0140	3.54 0.0524	1.32 0.3027	1.50 0.2035	1.19
Total fruit production	**22.05** **0.0000**	**232.66** **0.0000**	**1.10** **0.0000**	0.03 0.9328	**3.85** **0.0000**	**6.09** **0.0001**	0.53 0.1168	0.44 0.2919	0.59 0.2123	0.36 0.5595	0.44 0.2854	0.38

Table 3. Heritabilities calculated by generation and treatment, full data set. Boldface indicates significant values after a sequential Bonferroni correction. Top row: broad sense heritability, middle: standard errors, bottom: p-values.

	Bolting time	Leaf number at bolting	Rosette diameter	Set of first fruits	Shoot weight	Root weight	Number of lateral branches	Number of basal stems	Total fruit production
F1 Flooded 47 families	**0.83** **0.12** **0.00000**	**1.06** **0.10** **0.0000**	**0.63** **0.23** **0.0000**	0.15 0.22 0.5183	**0.31** **0.18** **0.0182**	**0.56** **0.11** **0.0000**	0.12 0.15 0.2522	0.31 0.22 0.0521	**0.41** **0.16** **0.0101**
F1Unflooded 47 families	**0.95** **0.15** **0.0000**	**1.11** **0.18** **0.0000**	**1.04** **0.13** **0.0000**	**0.17** **0.08** **0.0079**	**0.38** **0.11** **0.0000**	**0.66** **0.12** **0.0000**	**0.48** **0.16** **0.0000**	**0.42** **0.14** **0.0000**	**0.30** **0.10** **0.0018**
F3 Flooded 34 families	**1.19** **0.15** **0.0000**	**1.27** **0.15** **0.0000**	**0.69** **0.12** **0.0000**	**0.49** **0.22** **0.0100**	**0.17** **0.06** **0.0027**	**0.15** **0.11** **0.0366**	**0.39** **0.09** **0.0000**	**0.25** **0.13** **0.0028**	0.17 0.10 0.0449
F3 Unflooded 29 families	**1.02** **0.17** **0.0000**	**0.94** **0.20** **0.0000**	**0.91** **0.21** **0.0000**	**0.44** **0.14** **0.0017**	0.39 0.19 0.0575	**0.34** **0.13** **0.0091**	**0.27** **0.11** **0.0026**	-0.03 0.14 0.6338	0.14 0.10 0.0529

Table 4. Heritabilities for the sub data set including only accessions present in both treatments by the end of the selection experiment. Boldface indicates significant values after a sequential Bonferroni correction. Top row: broad sense heritability, middle: standard errors, bottom: p-values.

	Bolting time	Leaf number at bolting	Rosette diameter	Set of first fruits	Shoot weight	Root weight	Number of lateral branches	Number of basal stems	Total fruit production
F1 Flooded 21 families	**0.63** **0.14** **0.0000**	**0.95** **0.22** **0.0000**	**0.68** **0.31** **0.0267**	0.14 0.26 0.6500	**0.34** **0.22** **0.0212**	**0.66** **0.16** **0.0035**	-0.11 0.09 0.8634	0.48 0.30 0.0311	0.42 0.19 0.0342
F1Unflooded 21 families	**0.92** **0.17** **0.0000**	**1.47** **0.20** **0.0000**	**1.13** **0.21** **0.0000**	0.26 0.14 0.0162	**0.38** **0.16** **0.0051**	-0.01 0.07 0.6644	-0.03 0.05 0.8124	**0.47** **0.21** **0.0133**	0.11 0.10 0.1483
F3 Flooded 21 families	**1.16** **0.18** **0.0000**	**1.22** **0.19** **0.0000**	**0.70** **0.15** **0.0000**	0.49 0.23 0.02222	**0.19** **0.05** **0.0084**	-0.03 0.09 0.5833	-0.10 0.06 0.9471	**0.34** **0.14** **0.0044**	0.13 0.09 0.0695
F3 Unflooded 21 families	**1.01** **0.19** **0.0000**	**0.93** **0.22** **0.0000**	**0.95** **0.22** **0.0013**	**0.44** **0.16** **0.0023**	0.44 0.20 0.0532	-0.01 0.05 0.6445	-0.01 0.04 0.571	**0.36** **0.17** **0.0124**	0.18 0.14 0.0733

Table 5. Regression analysis of the relationship between plastic traits and reproductive fitness in the first generation of the selection experiment. Boldface indicates significant effects at α=0.05.

	Treatment	*Effect*	*Std Coef*	*t*	*P(2 Tail)*
Linear terms	**Flooded F1**	Leaf number at bolting	-0.14	-3.79	**0.0002**
		Rosette diameter	0.14	3.31	**0.0011**
		Set of first fruits	0.09	2.99	**0.0031**
		Shoot fresh weight	0.71	13.00	**0.0000**
		Root fresh weight	0.04	0.86	0.3885
		Lateral branches No	0.11	3.60	**0.0004**
		Basal stems No	0.10	2.90	**0.0041**
	Non-flooded F1	Leaf number at bolting	-0.26	-5.47	**0.0000**
		Rosette diameter	0.33	5.47	**0.0000**
		Set of first fruits	0.10	3.37	**0.0009**
		Shoot fresh weight	0.43	9.15	**0.0000**
		Root fresh weight	0.12	2.60	**0.0099**
		Lateral branches No	0.10	3.19	**0.0016**
		Basal stems No	0.26	6.98	**0.0000**
Quadratic terms	**Flooded F1**	(Leaf number at bolting)^2	0.07	0.72	0.4715
		(Rosette diameter)^2	0.84	2.20	**0.0288**
		(Set of first fruits)^2	-0.35	-2.00	**0.0468**
		(Shoot fresh weight)^2	-0.28	-1.65	0.1009
		(Root fresh weight)^2	-0.04	-1.11	0.2693
		(Lateral branches No)^2	-0.18	-1.92	0.0565
		(Basal stems No)^2	0.15	2.24	**0.0260**
	Non-flooded F1	(Leaf number at bolting)^2	0.12	1.11	0.2663
		(Rosette diameter)^2	-1.48	-3.08	**0.0023**
		(Set of first fruits)^2	-0.41	-0.73	0.4632
		(Shoot fresh weight)^2	0.21	2.55	**0.0112**
		(Root fresh weight)^2	0.00	-0.02	0.9869
		(Lateral branches No)^2	-0.07	-0.55	0.5821
		(Basal stems No)^2	-0.23	-2.15	**0.0328**

Table 6. Regression analysis of the relationship between plastic traits and reproductive fitness in the third generation of the selection experiment. Boldface indicates significant effects at α=0.05

	Treatment	*Effect*	*Std Coef*	*t*	*P(2 Tail)*
Linear terms	**Flooded F3**	Leaf number at bolting	-0.18	-4.96	**0.0000**
		Rosette diameter	0.00	0.03	0.9762
		Set of first fruits	0.04	1.71	0.0878
		Shoot fresh weight	0.90	20.12	**0.0000**
		Root fresh weight	0.03	0.99	0.3222
		Lateral branches No	0.12	3.96	**0.0001**
		Basal stems No	0.09	3.20	**0.0015**
	Non-flooded F3	Leaf number at bolting	-0.22	-5.57	**0.0000**
		Rosette diameter	0.08	1.67	**0.0000**
		Set of first fruits	0.11	4.21	0.0971
		Shoot fresh weight	0.67	15.57	**0.0000**
		Root fresh weight	0.03	0.81	0.4207
		Lateral branches No	0.14	5.25	**0.0000**
		Basal stems No	0.21	6.75	**0.0000**
Quadratic terms	**Flooded F3**	(Leaf number at bolting)^2	-0.37	-3.44	**0.0007**
		(Rosette diameter)^2	0.48	1.39	0.1664
		(Set of first fruits)^2	-0.26	-1.00	0.3195
		(Shoot fresh weight)^2	0.02	0.13	0.8939
		(Root fresh weight)^2	-0.01	-0.22	0.8263
		(Lateral branches No)^2	-0.11	-1.43	0.1552
		(Basal stems No)^2	0.06	1.35	0.1798
	Non-flooded F3	(Leaf number at bolting)^2	0.00	-0.04	0.9712
		(Rosette diameter)^2	-0.29	-0.97	0.3350
		(Set of first fruits)^2	-0.03	-0.10	0.9214
		(Shoot fresh weight)^2	0.29	3.18	**0.0017**
		(Root fresh weight)^2	-0.03	-1.30	0.1957
		(Lateral branches No)^2	-0.13	-1.82	0.0700
		(Basal stems No)^2	-0.17	-2.23	**0.0264**

Table 7. Correlation between eigenvectors expressed in the two treatments during the first generation. Boldfaced are correlations statistically significant at α=0.05 after table-wide Bonferroni corrections.

Correlation between vectors	Vector 1	Vector 2	Vector 3	Vector 4	Vector 5	Vector 6	Vector 7	Vector 8	Vector 9
Pearson's coefficient (p-value)	**0.86 (0.0026)**	**-0.87 (0.0026)**	0.63 (0.0697)	-0.73 (0.0256)	-0.21 (0.5950)	-0.48 (0.1960)	-0.12 (0.7580)	**0.97 (0.0000)**	0.58 (0.1018)
Total variance explained for flooded F1	41.11%	20.52%	12.92%	9.36%	5.81%	4.34%	3.14%	1.9%	0.88%
Total variance explained for non-flooded F1	45.92%	19.55%	11.88%	9.27%	4.66%	3.66%	2.18%	1.61%	1.27%

Table 8. Correlation between eigenvectors expressed in the two treatments during the third generation. Boldfaced are correlations statistically significant at α=0.05 after table-wide Bonferroni corrections.

Correlation between vectors	Vector 1	Vector 2	Vector 3	Vector 4	Vector 5	Vector 6	Vector 7	Vector 8	Vector 9
Pearson's coefficient (p-value)	0.71 (0.0338)	**0.97 (0.0000)**	0.004 (0.9923)	0.39 (0.2958)	0.04 (0.9275)	0.40 (0.2843)	**0.89 (0.0012)**	**0.95 (0.0001)**	**-0.94 (0.0001)**
Total variance explained for flooded F3	38.25%	24.67%	12.75%	8.71%	8.35%	3.19%	2.43%	0.99%	0.65%
Total variance explained for non-flooded F3	44.65%	27.2%	9.71%	7.12%	4.49%	3.48%	1.47%	1.02%	0.86%

Table 9. Correlation between eigenvectors expressed in the two generations under non-flooded treatment. Boldfaced are correlations statistically significant at α=0.05 after table-wide Bonferroni corrections.

Correlation between vectors	Vector 1	Vector 2	Vector 3	Vector 4	Vector 5	Vector 6	Vector 7	Vector 8	Vector 9
Pearson's coefficient (p-value)	**0.93 (0.0002)**	**-0.85 (0.0038)**	0.27 (0.4880)	0.52 (0.1489)	**0.90 (0.0009)**	**-0.92 (0.0005)**	0.60 (0.0866)	0.78 (0.0126)	-0.79 (0.0113)
Total variance explained for non-flooded F1	45.92%	19.55%	11.88%	9.27%	4.66%	3.66%	2.18%	1.61%	1.27%
Total variance explained for non-flooded F3	44.65%	27.2%	9.71%	7.12%	4.49%	3.48%	1.47%	1.02%	0.86%

Table 10. Correlation between eigenvectors expressed in the two generations under flooded treatment. Boldfaced are correlations statistically significant at α=0.05 after table-wide Bonferroni corrections.

Correlation between vectors	Vector 1	Vector 2	Vector 3	Vector 4	Vector 5	Vector 6	Vector 7	Vector 8	Vector 9
Pearson's coefficient (p-value)	0.61 (0.0782)	**0.96 (0.0000)**	-0.07 (0.8529)	0.17 (0.6655)	-0.22 (0.5730)	-0.54 (0.1340)	-0.66 (0.0528)	**0.85 (0.0035)**	**0.98 (0.0000)**
Total variance explained for flooded F1	41.11%	20.52%	12.92%	9.36%	5.81%	4.34%	3.14%	1.9%	0.88%
Total variance explained for flooded F3	38.25%	24.67%	12.75%	8.71%	8.35%	3.19%	2.43%	0.99%	0.65%

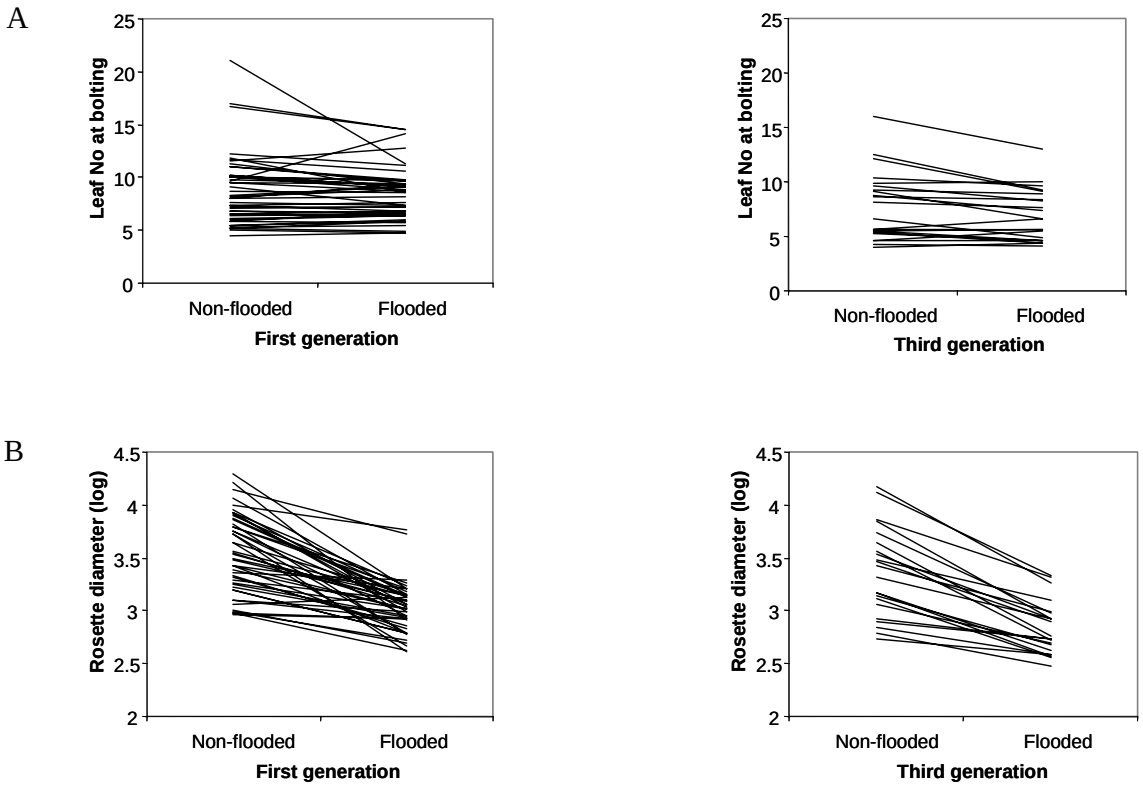
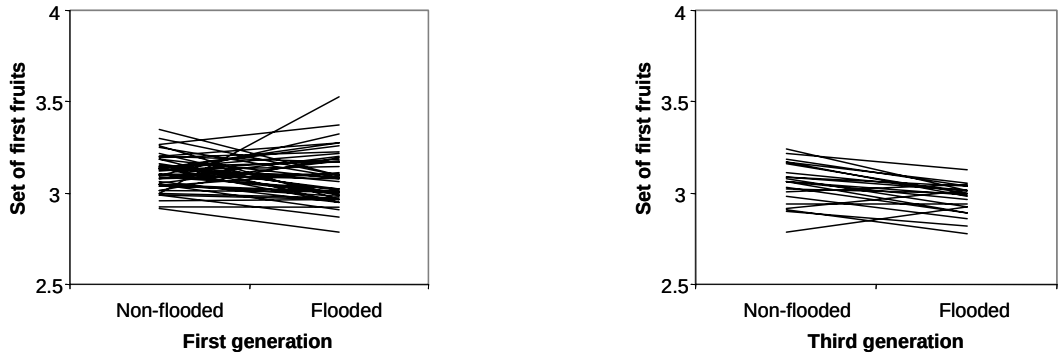


Figure 1. Reaction norms of genetic accessions that had representatives in both the first and last generation, under both environmental conditions. Each pair of plots shows the reaction norms for the same character in the first (left) and third (right) generation of the selection experiment.

C



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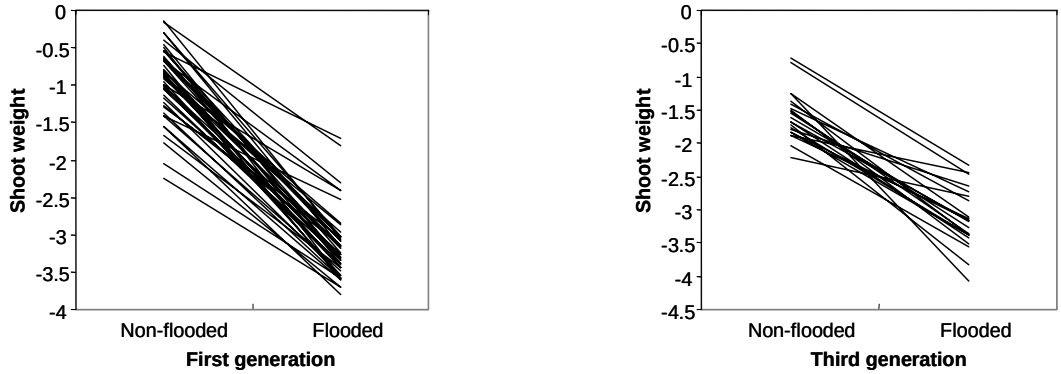

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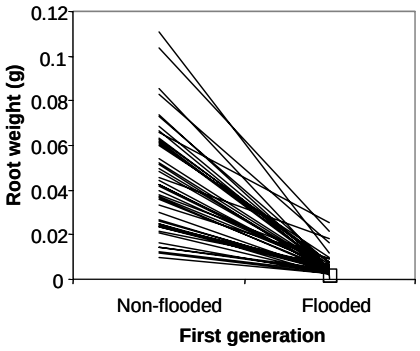

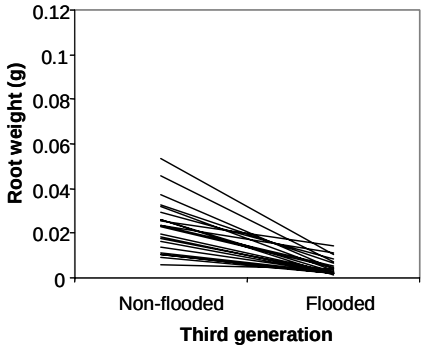

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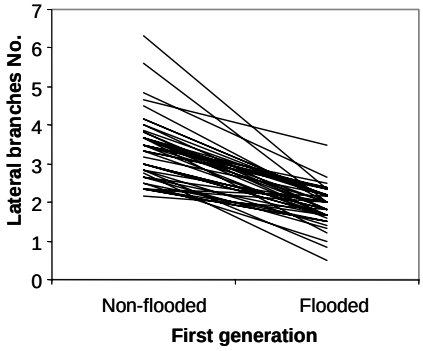

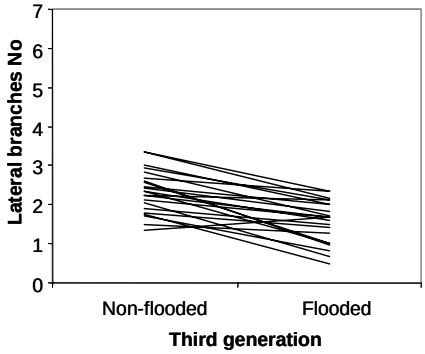

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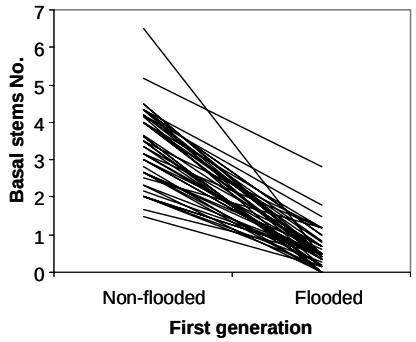

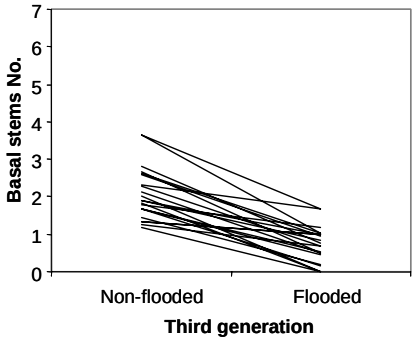

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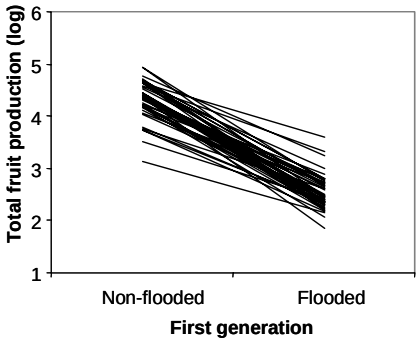

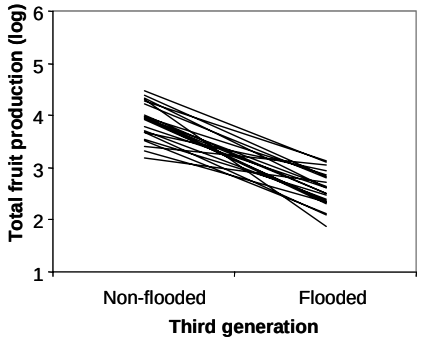

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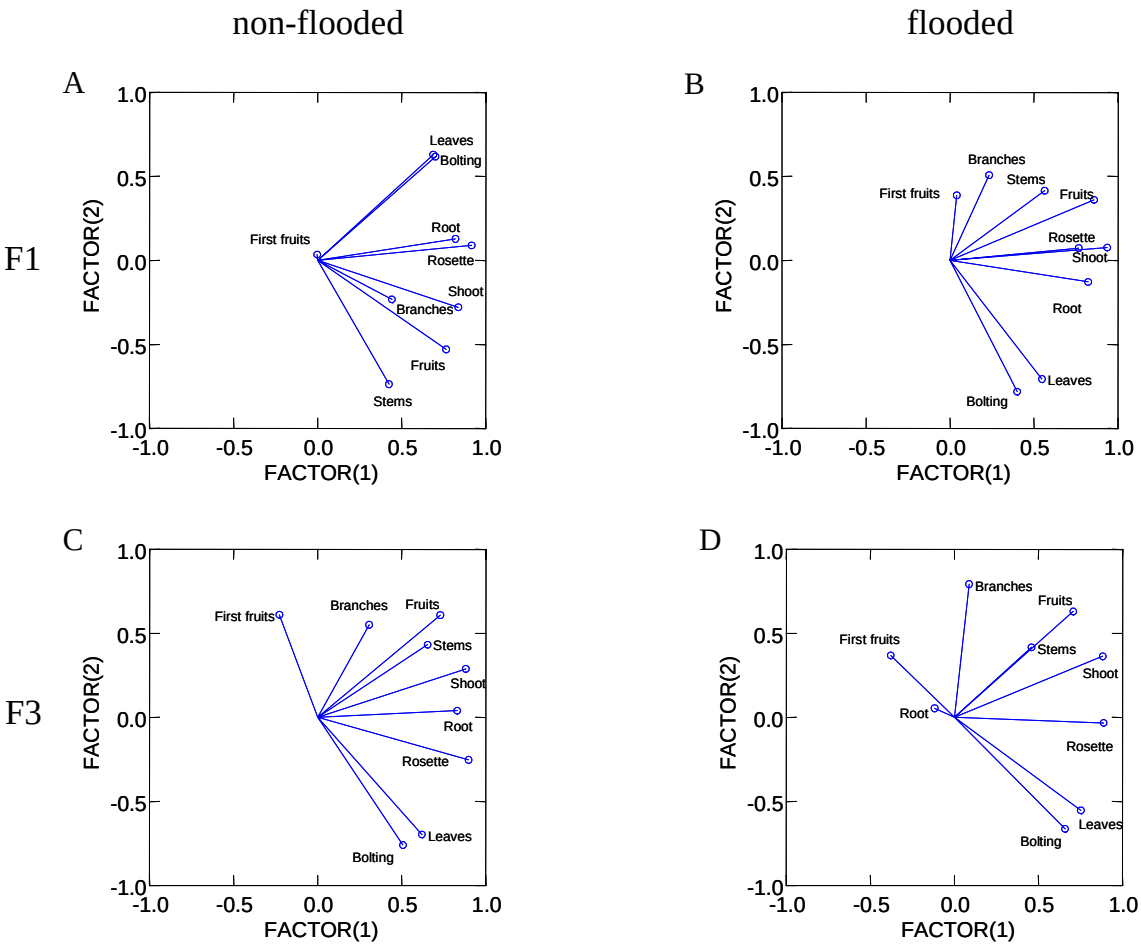

Figure 2. Principal components analyses of phenotypic integration as expressed under flooded (right) and non-flooded (left) conditions during the first (top row) and last (bottom row) generations of selection. The angles between vectors indicate the degree of independence of individual variables. Only the first two principal components are shown (see Tables 7-10 for the percentages of variance explained by each eigenvector).

PART IV:

Phenotypic integration in *Arabidopsis thaliana* (L.) Heynh.

Statement:
This part of the dissertation was submitted for publication to the American Journal of Botany.

All tables and figures are located in the Appendix IV.

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Keywords: *Arabidopsis thaliana*, flooding stress, light intensity, phenotypic integration, genetic covariances.

Running title: Phenotypic integration in Arabidopsis.

Introduction

In his article “Some of the evolutionary consequences of being a plant” Bradshaw (1972) discusses the implications of differences in the basic biology of plants and animals. Among the most obvious of such differences is the fact that plants are generally immobile during their lives (literally, rooted to their spot), and consequently have to cope with changes in their environment by different means than animals: instead of behavioral habitat selection, they must rely on some degree of phenotypic plasticity. Plasticity is the property of a genotype to produce different phenotypes in response to distinct environments (Schmalhausen, 1949). Different environments are also known to induce distinct patterns of character correlations (Schlichting and Levin, 1986), although it is far from clear to what extent this is the result of natural selection for “phenotypic integration” or of genetic or developmental constraints of one form or another.

Light and water are key environmental factors affecting plants’ life. Different aspects of light availability (quantity, daylength, spectral quality, angle of incidence) are perceived by specialized photoreceptors (Walters and Jennifer J.M. Rogers, 1999) that induce responses that are considered adaptive (Schmitt et al., 1995; Schmitt, 1997). Plasticity in leaf morphology induced by light quantity, for example, is one of the best known responses to environmental heterogeneity (Nunez-Olivera and J. Martinez-Abaigar, 1996). Water is also a highly variable abiotic factor affecting the life of all living organisms. For plants, both shortage (drought) and excess (waterlogging or flooding) of water are stressful and the two conditions elicit distinct coping mechanisms (Reader and Jalili, 1992; Armstrong and R. Brandle, 1994; Blom and Voesenek, 1996; Sperry, 2000; Zhang and T. Van Toai, 2000). Light and water availability are often coupled in nature in such a way that sites rich in light are usually characterized by a shortage of water while shady environments are more wet. It is the plastic response to this coupling of light quantity and water availability that we investigated in this study.

We have characterized genetic differentiation of a series of reaction norms to a full factorial combination of two levels of light availability (low and medium) and two levels of water conditions (flooded and non-flooded) in a collection of genotypes of the opportunistic weed *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae). *A. thaliana* is a model system widely used in molecular and physiological studies (Meyerowitz, 1989; Meinke, 1994; Convay and Poethig, 1997; Ellis and Elisabeth S. Dennis, 1999), and has recently received much attention from an ecological and evolutionary standpoint (Clauss and Aarssen, 1994b; Mauricio and Rausher, 1997; Li and Jun-Ichirou Suzuki, 1998; Pigliucci, 1998).

In this paper we address the following questions: 1. Is there genetic differentiation for trait means among genotypes representative of different populations when exposed to a combination of light and water availability? 2. Are there plasticity and variation for plasticity to combinations of light and water availability for the traits of interest? 3. Do plastic traits affect reproductive fitness, suggesting that they may be under selection depending on the environmental conditions actually experienced by these plants in the field (see Discussion for a rationale of studies of selection under controlled conditions)? 4. Do environmental changes affect the variance-covariance matrix relating different life history and morphological traits (phenotypic architecture), and if so to what extent?

It is important to realize that we are here comparing genotypes from a variety of geographically dissimilar accessions, which means that we are looking at the *outcome* of past evolutionary forces and how they shaped the degree of divergence for plasticity and integration that we observe today. This is in contrast to within-population studies whose major goal is to assess the current degree of genetic variation and the possible future responses of a population to evolutionary forces. As it has been pointed out for example by Armbruster and Schwaegerle (Armbruster and Schwaegerle, 1996), both levels of analyses are important to evolutionary theory because they address respectively medium and short term evolutionary phenomena. The former,

however, are far less often the goal of empirical investigations, a situation that we are attempting to correct (Pigliucci and Kolodynska, in press).

Materials and Methods

Bulk collections of seeds from 22 accessions of *Arabidopsis thaliana* (L.) Heynh. were obtained from the *Arabidopsis* Information Management System (AIMS at www.arabidopsis.org): CS0911, Estland (Germany); CS0913, Petergof (Russia); CS0917, Darmstadt (Czechoslovakia); CS0922, Hodja-Obi-Garm (Tadjikistan); CS0925, Litvania (Litvania); CS0932 Aberdeen (UK); CS1214, Guckingen (Germany); CS1226, Hilversum (Netherlands); CS1252, Vranov (Czechoslovakia); CS1282, Rodenbach (Germany); CS1604, Wietze (Germany); CS1630, Wildbad (Germany); CS1635, Canterbury (U.K.); CS3179, Graz (Austria); CS6023, Sedmouth (UK); CS6034, Bretagny (France); CS6041, Kelsterbach (Germany); CS6046, Koln (Germany); CS6068, Kent (UK); CS6105, Kelsterbach (Germany); CS6194, Blanes (Spain); CS6195, Wurzburg (Germany); and CS6731 Gluckingen, (Germany).

All accessions selected for our experiment represented early flowering populations of *A. thaliana* and had been bulk propagated at AIMS to maintain genetic variation. In order to minimize maternal effects and increase seed availability, we grew the material for one generation under controlled laboratory conditions of 16/8 hour of light/darkness at a room temperature of 23-25°C and provided bottom watering every other day to reduce mechanical interference.

These second generation seeds were used for a selection experiment on the response to flooding (Pigliucci and Kolodynska, submt.), and seeds from the non-stress environment of the third generation of that experiment were used for the current experiment. The genetic material used in this experiment was not significantly altered by the selection process because it occurred mainly by line sorting, with little chance for inter-genotypic recombinational events. Seeds were placed on a wet paper filter and cold treated for one week at 4°C in the refrigerator in order to

increase and homogenize germinability. Imbibed seeds were planted in a mixture of topsoil-coarse sand-turface (2-2-1 by volume) and placed under high intensity growth racks. Seedlings were thinned, leaving one plant per pot, when plants developed the first true leaves, and top-fertilized once a week for the first four weeks of the experiment with 2ml of NPK (11:11:11).

Measurements

The following *vegetative traits* were measured at the bolting stage, when the rosette begins to produce the flowering stem: 1) Bolting time (time from planting the seeds to the initiation of the main stem); 2) Rosette leaf number, quantifying meristem allocation to vegetative growth; and 3) Rosette diameter, a measure of plant size during the vegetative phase. *Plant architecture* traits were measured at the time of harvest, one week after maturation of the first fruits (manifested as opening of the siliques on the main inflorescence). The architecture traits were: 4) Number of lateral branches; 5) Above ground fresh weight (measuring plant allocation of resources to above ground structures), including the rosette, the main stem, branches, and fruits; 6) Number of bracts on the main stem; and 7) Total number of basal stems (allocation of resources to secondary meristems), including both elongated and non-elongated basal stems. If a given basal stem had opened inflorescences it was counted as an elongated stem, otherwise as a non-elongated one (which measures further *potential* reproductive success). *Reproductive traits* were measured after the plants set the first fruits: 8) Time of first reproduction, when the first seeds matured and the siliques started opening, counted as days from bolting (i.e., from the beginning of the reproductive phase); and 9) Total fruit production (reproductive fitness).

Experimental design and statistical analyses

Plants from each accession were randomly assigned to one of the four combinations of water and light treatment (low light / flooded, low light / non-flooded, medium light / flooded,

and medium light / non-flooded), with every accession represented by six replicates within each treatment. The total size of the experimental population was therefore 22 accessions by 4 treatments by 6 replicates = 528 plants. Individuals were placed in two growth racks with each rack housing three shelves, with four trays on each shelf. Combinations of the two environmental factors were applied in a two–way full factorial design. Each shelf housed both water level treatments and one light level, with the light treatments alternated across racks in such a way that the first growth rack housed two shelves with medium light and one with low light, and the second rack had two shelves with low light and one with medium light. The positions of the accessions were randomized within trays.

Measured variables deviating from normality or homoscedasticity were appropriately transformed (Sokal and Rohlf, 1995). We employed a nested mixed-model analysis of variance (split-plot design: SYSTAT, 2000) to estimate the significance of the following factors: A. Accession, testing the degree of genetic differentiation among accessions, all other things being equal. B. Light, estimating the overall degree of phenotypic plasticity in response to light. C. Water, estimating the overall degree of phenotypic plasticity in response to water. D. Light by Water, estimating overall phenotypic plasticity to combinations of light and water levels, regardless of the specific genetic background. E. Light by Accession, estimating genetic differentiation for plasticity to light among accessions. F. Water by Accession, estimating genetic differentiation for plasticity to water among accessions. G. Light by Water by Accession, testing for genetic differentiation for plasticity in response to combinations of both water and light availability. H. Shelf (nested within Rack), estimating the degree of micro-environmental variation attributable to the experimental setup.

Treatments were considered fixed effects, while Accession and Shelf(Rack) were treated as random effects. According to Sokal and Rohlf (1995), in a mixed-model ANOVA one needs to test the main effects over the corresponding interaction terms, if the latter are significant; the same goes for testing nested effects over the corresponding nesting factor. Otherwise, factors

were tested over the error mean square (this judicious use of conservative statistical tests is advocated by Sokal and Rohlf, and we consider it better than always testing over interactions or lower-level effects, even when these are not significant). Given the high number of multiple comparisons (several traits analyzed simultaneously), we used a sequential Bonferroni correction to adjust the nominal α-values for the ANOVAs across rows in Table 1 (again, this correction is moderately conservative, as opposed to a straight Bonferroni, which tends to overcorrect for type II errors: Rice, 1989). Reaction norms were plotted for all traits showing significant genotype by environment interaction to visualize the patterns of genotypic response to changes in the environment.

Regression analyses were used to obtain information on the type of selection (directional or stabilizing) that was operating on the traits showing plasticity under the conditions we employed. In order to test whether the phenotypic correlations among these traits were affected by environmental changes, we performed principal components analyses and vector correlation analyses, comparing factor loadings from the different combinations of light and water levels.

Results

Genetic differentiation and phenotypic plasticity

The analysis of variance showed widespread variation of character means among accessions (seven out of nine traits were characterized by a significant Accession effect in Table 1, Fig. 1). Six of the nine traits showed plasticity in response to light levels (timing of bolting, leaf number at bolting time, number of bracts, shoot weight, number of lateral branches, and number of basal stems), and five out of nine traits showed plasticity to water (leaf number, bract number, shoot weight and lateral and basal branching). All traits displaying plasticity to water

were also plastic in response to light, but not vice versa. Three traits showed a complex type of phenotypic plasticity which depended on the interaction between water and light levels (rosette diameter, shoot weight, and total fruit production: Figure 1e,f,i,j,o,p).

Leaf number at bolting time and number of bracts (Fig. 1a,b and g,h) showed genetic differentiation for plasticity in reaction to light. On the other hand, there was no genetic differentiation for plasticity to water among our accessions. None of the traits measured showed significant genetic differentiation for combinations of light and water plasticity. As expected, there was widespread variation of character means attributable to micro-environmental effects generated by the experimental setup.

In general, the differentiation of the reaction norms of different traits fell into three classes (Fig. 1): some traits showed plasticity while the environmental means were not different (e.g., leaf number and to a lesser extent bract number, as well as basal stem and fruit production); other characters showed markedly lower means under medium than low light, regardless of the water treatment (e.g., bolting time, rosette diameter, shoot weight, and lateral branching); regardless of their response to light, some traits displayed a reaction to water stress (flood), such as rosette diameter, bract number (to a lesser extent), shoot weight, lateral branching, basal stems, and fruit production.

Selection acting on plastic traits

Seven of the eight traits measured showed plasticity of one sort or another and we explored their relationship with reproductive fitness via multiple regression analyses. Bolting time, leaf number at bolting, and shoot weight were under directional selection under medium light intensity and flooded water conditions, which favored earlier bolting and an increase in leaf number and shoot weight (Table 2). Only shoot weight was under apparent disruptive selection

under the same environmental conditions, although an examination of the actual distribution of the data (not shown) revealed that this was a case of non linear directional selection instead.

Number of lateral branches, number of basal stems, and shoot weight were under directional selection for an increase in the trait value under medium light intensity and non-flooded water conditions (Table 2). None of the measured traits showed a statistically significant quadratic regression coefficient under these conditions.

Shoot weight was the only character under significant directional selection under low light intensity and flooded water regime (Table 3). Under the same environment, bract number was under apparent disruptive selection and a visual inspection of the data (not shown) did indeed support the possibility of selection favoring high and low numbers of bracts but not medium numbers.

Bolting time, number of lateral branches, rosette diameter, and shoot weight were under directional selection under low light intensity and non-flooded water conditions, with selection favoring earlier bolting and increased expression of the other traits (Table 3). Leaf number at bolting was the only trait under apparent disruptive selection, a conclusion weakly supported by a visual inspection of the data (details not shown).

Changes in multivariate structure induced by the environment

We used principal components and vector correlation analyses to explore the degree of similarity between phenotypic architectures as expressed under different combinations of light and water treatments. A visual inspection of the plots of the factor loadings on the first two eigenvectors revealed a high degree of similarity in the orientation of the multivariate vectors under both water treatments and Medium Light (Fig. 2a,b). Bolting time, number of bracts, number of leaves and timing of the first reproduction were closely associated to each other under Non-flooded conditions, while fruit number, shoot weight and rosette diameter constituted a tight

cluster under Flooded conditions. These clusters of traits were rotated by 180 degree between the Flooded and Non-Flooded treatments, reflecting an overall high degree of similarity of the pattern of integration between these two environments.

Similar groupings of traits appeared under Low Light (Fig. 2c,d), except that the time of first reproduction was now more prominent and clearly associated with branching, shoot weight and other reproductive traits.

Vector correlation analyses showed that under both medium and low light the first three vectors expressed under flooded and non-flooded conditions shared an almost identical structure (Tables 4 and 5). These vectors explained a bit more than 70% of the total phenotypic variance. The situation was different when we compared the matrices expressed under contrasting light levels while holding water constant (Tables 6 and 7). Here all major eigenvectors (except for the second one under both conditions) were independent of each other, indicating a higher degree of rearrangement of the phenotypic correlation matrix in response to light availability.

Discussion

Phenotypic evolution is a complex field of study that involves an understanding of the amount of genetic differentiation of characters, their lability to environmental conditions, their association with fitness, as well as their relationship with other aspects of the phenotype (Schlichting and Pigliucci, 1998). In this study we attempted to characterize phenotypic divergence among accessions of *A. thaliana* and to study how the correlations among traits were affected by changes in two important environmental factors, water and light availability.

We investigated individual traits and their relationship with reproductive fitness, as well as the multivariate patterns of phenotypic integration and their lability to environmental change (Schlichting, 1989). There is a considerable interest in the study of phenotypic and genetic

correlations because of their relevance to evolutionary theory (Roff and Mouseau, 1999), especially with regard to the validity of the assumptions embedded in quantitative genetics models of evolutionary change (Turelli, 1988; Pigliucci and Schlichting, 1997) and to our understanding of multivariate phenotypic evolution (Schlichting and Pigliucci, 1998).

Genetic differentiation and phenotypic plasticity

We have observed widespread genetic differentiation among accessions for trait means when grown in a combination of water availability and light quantity, in agreement with several previous studies on this species (Aarssen and Clauss, 1992; Clauss and Aarssen, 1994a; Pigliucci et al., 1995). We have also observed widespread plasticity for both light and water, when considered separately. Interestingly, however, few traits showed genetic differentiation for plasticity, and only in response to changes in light regime.

This high degree of genetic differentiation for trait means but not for plasticity is consistent with what we know of the species' life history and autoecology: *A. thaliana* flowers in the spring (when light is abundant) and is probably exposed to random fluctuations in flooding regimes, which depend on the local geography. Under these conditions these plants are not expected to evolve adaptive plasticity to water, but rather genetic specialization for whatever water regime they encounter more often (Pigliucci, 2001).

A similar explanation applies to plasticity and genetic differentiation of light responses: *A. thaliana* is a colonizer of mostly open habitats, where there is little or no shade. As shown by Bell (1997) with work on *Chlamydomonas*, such coarse-grained environmental variation promotes genetic differentiation but not plasticity. It is also worth recalling that at least some of the observed genetic differentiation among *A. thaliana*'s populations (which is observed not only for quantitative, but also for molecular traits: Innan et al., 1997; Loridon et al., 1997; Breyne et al., 1999; Erschadi et al., 2000) is probably due to historical patterns of diversification after the

last glaciation (Sharbel et al., 2000) and a significant role for natural selection to explain genetic differentiation in this species has still to be demonstrated.

Generally speaking, all traits showed the highest values when expressed under non-flooded water treatment and low light intensity. Such pattern can be explained by a combination of possibly adaptive responses to low light availability (increased leaf size and bract number to compensate for a lower photosynthetic output) and a generally healthy growth rate coupled with the absence of water stress (resulting in larger and more branched plants). Accordingly, this lead to the highest reproductive output among the four combinations of environmental conditions.

Flooded conditions and medium light intensity probably represented a quite un-natural combination of treatments for *A. thaliana* (which does not typically grow on wetlands), so it is not surprising that our accessions showed signs of severe stress expressed as the lowest measured trait values in this experiment. This was likely the combined result of the novelty and stressfulness of that particular treatment, with the plants unable to cope with it because of lack of past selection under similar conditions and intrinsic limits to their physiology.

Recent work on the molecular genetics of *Arabidopsis* responses to water stress may also help interpreting our and future results of organismal studies in this species. Several authors have implicated cytokinin and abscisic acid production in the regulation of osmotic signal transduction and even in adaptation to flood conditions (Ishitani et al., 1997; Zhang et al., 2000), although most work in this area has been done on drought rather than flood response. Similarly, the molecular work on the basis of light responses has largely concentrated on shade avoidance and to a lesser extent photoperiod and phototropism, rather than response to irradiance per se (Whitelam and Devlin, 1998). The latter seem to be mediated by phytochrome A at low irradiance levels (Botto et al., 1996) and by blue-sensitive photoreceptors at high irradiance levels (Liscum and Hangarter, 1994), but so far little is known about the transduction pathways involved in plasticity to light quantity.

Selection acting on plastic traits

Regression analyses performed on traits showing plasticity or genetic differentiation for plasticity detected surprisingly little evidence of linear or quadratic selection, with earlier bolting (i.e., a shorter vegetative phase) favored under medium light / flooded and low light / non flooded. Selection for early flowering has been detected in several occasions in this species, under both controlled and field conditions (Callahan and Pigliucci, in press) and has probably shaped the overall ecological strategy of this weed, which is a weak competitor of mostly ruderal habitats.

As far as other characters are concerned, perhaps surprisingly we found more aspects of *A. thaliana*'s phenotype to be under selection in the non-flooded than in the flooded treatments. In particular, we observed a relationship between reproductive fitness and increased lateral branching, basal branching, shoot weight and rosette diameter (depending on the light treatment) in the non-flooded environment, while only increased shoot weight seemed to be beneficial under flooded conditions. In general, these results point toward larger plants being associated with enhanced reproductive fitness under non-stressful conditions (although this was much more true for reproductive than for vegetative characters) and a rather minimalist phenotype under stress.

While one could object that studying selection in artificial environments is not particularly informative, we suggest that on the contrary this may yield more clear results than studies conducted in natural environments, simply because the latter are confounded by a large number of factors that tend to cancel each other and yield highly variable and weak estimates of selection coefficients (Hoekstra et al., 2001; Kingsolver et al., 2001). Of course, for studies under controlled conditions to be ecologically informative they have to be carried out under a simplified set of realistic environments, which represents a challenge in the case of the many species for which we don't have an accurate characterization of the autoecology. This means that more research on *A. thaliana*'s ecology (e.g., Callahan and Pigliucci, in press) is much needed,

especially in light of the status as a "model system" of this species in both molecular and organismal studies.

Changes in multivariate structure induced by the environment

Classical studies in evolutionary ecology tended to focus on variation in single characters (with some notable exceptions: Berg, 1960; Clausen and Hiesey, 1960), but recently there has been an increase in interest in the co-variation among characters and its consequences for phenotypic evolution (Steppan, 1997; Arnold and Phillips, 1999; Phillips and Arnold, 1999; Waldman and Anderson, 2000). We were particularly interested in the relationship between phenotypic integration (assessed by the pattern of character correlations) and environmental variation, i.e., in how the environment can alter the patterns of phenotypic correlations among traits, which in our case represent genetic differentiation among accessions.

Both a visual inspection of the correlation matrices obtained under either water environment (not shown) and the principal components analyses showed a fairly high degree of similarity between the major aspects of the character architecture as expressed under the two water treatments, regardless of the light conditions. On the contrary, when we compared the matrices expressed under the two light conditions we observed much less similarity, independently of the water treatment. These mixed results exemplify how much studies of phenotypic integration depend not only on the particular genotypes studied (Pigliucci and Hayden, in press), but also on the environment(s) to which those genotypes are exposed.

Several authors have observed a certain degree of conservativeness of phenotypic integration matrices among populations, for example in the case of a study by Arnold and Phillips (1999) on coastal/inland divergence in garter snakes. Roff and Mousseau (1999) have reviewed the literature on divergence of genetic correlations across different taxonomic levels and found that the results are mixed, as one would expect considering the heterogeneity of methods, types of

characters, and taxa that have been employed and sampled so far. Future work will have to address the obvious problem that phenotypic or genetic "architectures" can be a highly heterogeneous category depending on the specific traits and organisms being considered.

The field of multivariate phenotypic evolution is also plagued by methodological problems. Roff (2000), for example, has compared various methods for examining multivariate genetic/phenotypic divergence and found that no single method yields satisfactory results. Our own experience with the currently popular common principal components (CPC) analyses (Phillips and Arnold, 1999; Waldmann and Andersson, 2000) is actually rather unsatisfying. Both in the case of the current study (not shown) and in other occasions (Pigliucci and Kolodynska, in press) we discovered that CPCs tend to be too sensitive and yield a verdict of no similarity among matrices even when it is obvious by both visual inspection and other methods (Mantel tests, vector correlation analyses) that there is in fact a high degree of overlap between matrices. This problem of finding satisfactory statistical methods to quantify changes in phenotypic integration (see also: Smouse et al., 1986; Cowley and Atchley, 1992; Shaw, 1992) is perhaps the major stumbling block against progress in this important field of inquiry into phenotypic evolution.

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Appendix IV

Table 1. Analysis of variance showing Mean Squares and associated p-values. Statistically significant effects after a table-wide Bonferroni correction are boldfaced. d.f. indicates the degrees of freedom associated with each factor. Transformations used to achieve normality are indicated in parenthesis next to the names of the traits.

Trait	Accession (21 d.f.) (A)	Light (1 d.f.) (L)	Water (1 d.f.) (W)	L×W (1 d.f.)	L×A (21 d.f.)	W×A (21 d.f.)	W×A×L (21 d.f.)	Shelf(Rack) (4 d.f.)	Error (330-368 df)
Bolting time	**391.84 (0.0000)**	**3832.73 (0.0000)**	27.03 (0.1839)	4.10 (0.6044)	19.93 (0.1663)	13.67 (0.5959)	18.70 (0.2251)	**617.13 (0.0000)**	15.25
Leaf number at bolting	**76.72 (0.0000)**	**55.91 (0.0000)**	**136.54 (0.0000)**	8.65 (0.0659)	**9.46 (0.0000)**	3.57 (0.1124)	2.71 (0.3820)	7.80 (0.0165)	2.54
Rosette diameter (log)	**0.99 (0.0000)**	28.36 (0.1617)	28.81 (0.1605)	**1.91 (0.0002)**	0.20 (0.0675)	0.17 (0.2049)	0.14 (0.4231)	**3.92 (0.0000)**	0.13
Bract number	**6.21 (0.0000)**	**14.43 (0.0000)**	**20.26 (0.0000)**	0.13 (0.6342)	**1.54 (0.0001)**	0.57 (0.4381)	0.59 (0.3941)	1.99 (0.0076)	0.56
Set of first fruits	**36.78 (0.0000)**	1432.91 (0.1314)	7.59 (0.2980)	62.79 (0.0029)	13.81 (0.0070)	8.85 (0.1947)	6.33 (0.5829)	**253.42 (0.0000)**	6.99
Shoot weight (log)	1.78 (0.0513)	**120.94 (0.0000)**	**226.54 (0.0000)**	**15.82 (0.0000)**	0.76 (0.0751)	0.34 (0.8661)	0.86 (0.0296)	**20.05 (0.0000)**	0.51
Number of lateral branches	**2.83 (0.0000)**	**116.01 (0.0000)**	**54.50 (0.0000)**	0.03 (0.8594)	1.43 (0.0175)	0.86 (0.3664)	0.98 (0.2223)	**22.57 (0.0000)**	0.80
Number of basal stems	**2.17 (0.0000)**	**7.30 (0.0005)**	**73.85 (0.0000)**	1.52 (0.1101)	1.17 (0.0070)	0.55 (0.5562)	0.90 (0.0662)	**2.81 (0.0010)**	0.59
Total fruit production	0.54 (0.6023)	17.26 (0.4068)	111.64 (0.1809)	**9.52 (0.0000)**	0.50 (0.0751)	0.25 (0.7910)	0.61 (0.0173)	**4.51 (0.0000)**	0.34

Table 2. Regression analysis of the relationship between plastic traits and reproductive fitness under Medium Light. Statistically significant effects at α=0.05 are boldfaced.

	Treatment	*Effect*	*Stand. Coeff.*	*t*	*P(2 Tail)*
Linear terms	**Flooded**	**Bolting time**	**-0.14**	**-2.14**	**0.0350**
		Leaf number at bolting	**0.21**	**2.54**	**0.0125**
		Bract number	-0.08	-1.56	0.1208
		Lateral branches	0.09	1.62	0.1085
		Basal stems	0.08	1.18	0.2422
		Rosette diameter	-0.08	-1.00	0.3201
		Shoot weight	**0.79**	**7.93**	**0.0000**
	Non-flooded	Bolting time	-0.04	-0.67	0.5029
		Leaf number at bolting	0.01	0.21	0.8361
		Bract number	-0.03	-0.60	0.5477
		Lateral branches	**0.16**	**3.13**	**0.0022**
		Basal stems	**0.14**	**2.81**	**0.0059**
		Rosette diameter	0.02	0.20	0.8411
		Shoot weight	**0.75**	**7.32**	**0.0000**
Quadratic terms	**Flooded**	$(\text{Bolting time})^2$	0.12	0.28	0.7823
		$(\text{Leaf number at bolting})^2$	-0.30	-0.99	0.3268
		$(\text{Bract number})^2$	0.05	0.30	0.7661
		$(\text{Lateral branches})^2$	0.17	0.96	0.3381
		$(\text{Basal stems})^2$	-0.07	-0.50	0.6215
		$(\text{Rosette diameter})^2$	-0.35	-1.14	0.2555
		$(\text{Shoot weight})^2$	**1.45**	**4.03**	**0.0001**
	Non-flooded	$(\text{Bolting time})^2$	-0.26	-0.74	0.4594
		$(\text{Leaf number at bolting})^2$	0.28	0.96	0.3372
		$(\text{Bract number})^2$	0.27	1.45	0.1510
		$(\text{Lateral branches})^2$	-0.10	-0.78	0.4375
		$(\text{Basal stems})^2$	-0.02	-0.17	0.8672
		$(\text{Rosette diameter})^2$	0.10	0.18	0.8565
		$(\text{Shoot weight})^2$	0.34	1.08	0.2822

Table 3. Regression analysis of the relationship between plastic traits and reproductive fitness under Low Light. Statistically significant effect at α=0.05 are boldfaced.

	Treatment	*Effect*	*Stand. Coeff.*	*t*	*P(2 Tail)*
Linear terms	**Flooded**	Bolting time	-0.21	-1.71	0.0907
		Leaf number at bolting	0.03	0.24	0.8115
		Bract number	0.08	0.94	0.3510
		Lateral branches	0.05	0.51	0.6086
		Basal stems	0.12	1.40	0.1643
		Rosette diameter	0.16	1.13	0.2625
		Shoot weight	**0.57**	**4.16**	**0.0001**
	Non-flooded	**Bolting time**	**-0.29**	**-2.19**	**0.0315**
		Leaf number at bolting	0.20	1.52	0.1327
		Bract number	-0.07	-0.61	0.5423
		Lateral branches	**0.27**	**2.65**	**0.0099**
		Basal stems	0.10	0.95	0.3459
		Rosette diameter	**0.30**	**2.33**	**0.0230**
		Shoot weight	**0.44**	**3.54**	**0.0007**
Quadratic terms	**Flooded**	$(\text{Bolting time})^2$	0.82	1.15	0.2521
		$(\text{Leaf number at bolting})^2$	0.20	0.32	0.7500
		$(\text{Bract number})^2$	**1.26**	**3.04**	**0.0032**
		$(\text{Lateral branches})^2$	0.06	0.28	0.7786
		$(\text{Basal stems})^2$	0.20	1.01	0.3161
		$(\text{Rosette diameter})^2$	-1.71	-1.45	0.1500
		$(\text{Shoot weight})^2$	0.76	1.09	0.2803
	Non-flooded	$(\text{Bolting time})^2$	-0.83	-1.09	0.2788
		$(\text{Leaf number at bolting})^2$	**1.15**	**2.30**	**0.0247**
		$(\text{Bract number})^2$	0.55	1.11	0.2713
		$(\text{Lateral branches})^2$	-0.10	-0.39	0.7011
		$(\text{Basal stems})^2$	-0.20	-0.91	0.3649
		$(\text{Rosette diameter})^2$	-1.29	-0.93	0.3557
		$(\text{Shoot weight})^2$	0.79	1.35	0.1817

Table 4 . Correlation between eigenvectors expressed under the two water regimes when light was kept at a medium level. Boldface indicates correlations significant at α=0.05 after a Bonferroni correction.

Correlation between vectors	Vector 1	Vector 2	Vector 3	Vector 4	Vector 5	Vector 6	Vector 7	Vector 8	Vector 9
Pearson's coefficient (p-value)	**0.82 (0.0071)**	**-0.91 (0.0007)**	**0.90 (0.0009)**	0.43 (0.2477)	0.05 (0.8950)	-0.18 (0.6523)	-0.60 (0.0876)	0.61 (0.0820)	**-0.95 (0.0001)**
Total variance explained for Flooded	46.36%	15.05%	13.23%	9.92%	5.24%	3.70%	3.18%	2.35%	0.96%
Total variance explained for Non-flooded	41.98%	20.46%	11.13%	8.22%	7.14%	6.10%	2.50%	1.75%	0.71%

Table 5 . Correlation between eigenvectors expressed under the two water regimes when light was kept at a low level. Boldface indicates correlations significant at α=0.05 after a Bonferroni correction.

Correlation between vectors	Vector 1	Vector 2	Vector 3	Vector 4	Vector 5	Vector 6	Vector 7	Vector 8	Vector 9
Pearson's coefficient (p-value)	**0.91 (0.0007)**	**0.96 (0.0000)**	**0.60 (0.0010)**	0.08 (0.0874)	0.18 (0.8307)	0.56 (0.6507)	-0.33 (0.1154)	-0.37 (0.3812)	0.91 (0.3232)
Total variance explained for Flooded	34.49%	25.56%	12.45%	8.38%	6.56%	5.34%	3.44%	2.46%	1.33%
Total variance explained Non-flooded	38.21%	29.37%	10.84%	6.96%	4.08%	3.48%	3.46%	2.07%	1.53%

Table 6 . Correlation between eigenvectors expressed under the two light levels when the water treatment was flooded. Boldface indicates correlations significant at α=0.0 after a Bonferroni correction.

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Correlation between vectors	Vector 1	Vector 2	Vector 3	Vector 4	Vector 5	Vector 6	Vector 7	Vector 8	Vector 9
Pearson's coefficient (p-value)	0.47 (0.2013)	**0.87 (0.0023)**	0.78 (0.0122)	0.68 (0.0455)	-0.33 (0.3901)	0.26 (0.5029)	0.46 (0.2166)	0.61 (0.0811)	**0.88 (0.0019)**
Total variance explained for Medium Light	46.36%	15.05%	13.23%	9.92%	5.24%	3.70%	3.18%	2.35%	0.96%
Total variance explained for Low Light	34.49%	25.56%	12.45%	8.38%	6.56%	5.34%	3.44%	2.46%	1.33%

Table 7. Correlation between eigenvectors expressed under the two light levels when the water treatment was non-flooded. Boldface indicates correlations significant at α=0.0 after a Bonferroni correction.

Correlation between vectors	Vector 1	Vector 2	Vector 3	Vector 4	Vector 5	Vector 6	Vector 7	Vector 8	Vector 9
Pearson's coefficient (p-value)	0.80 (0.0102)	**-0.85 (0.0035)**	0.76 (0.0178)	0.70 (0.0346)	0.14 (0.7276)	0.01 (0.9897)	0.44 (0.2419)	0.35 (0.3632)	0.26 (0.4939)
Total variance explained for Medium Light	41.98%	20.46%	11.13%	8.22%	7.14%	6.10%	2.50%	1.75%	0.71%
Total variance explained for Low Light	38.21%	29.37%	10.84%	6.96%	4.08%	3.48%	3.46%	2.07%	1.53%

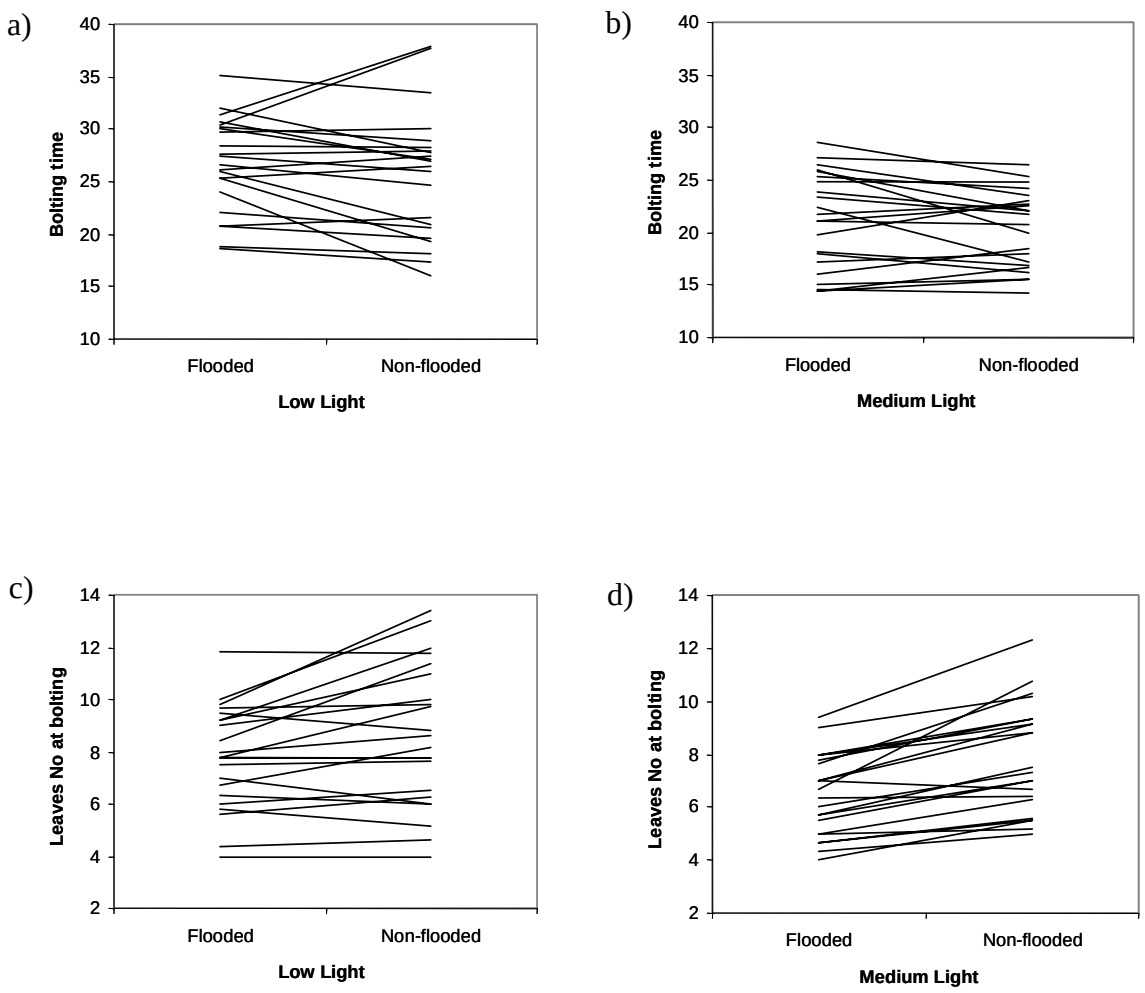

Figure 1. Reaction norms of genetic accessions under combinations of two environmental factors. Each pair of plots shows the reaction norms to water for the same character under low (left) and high (right) light conditions.

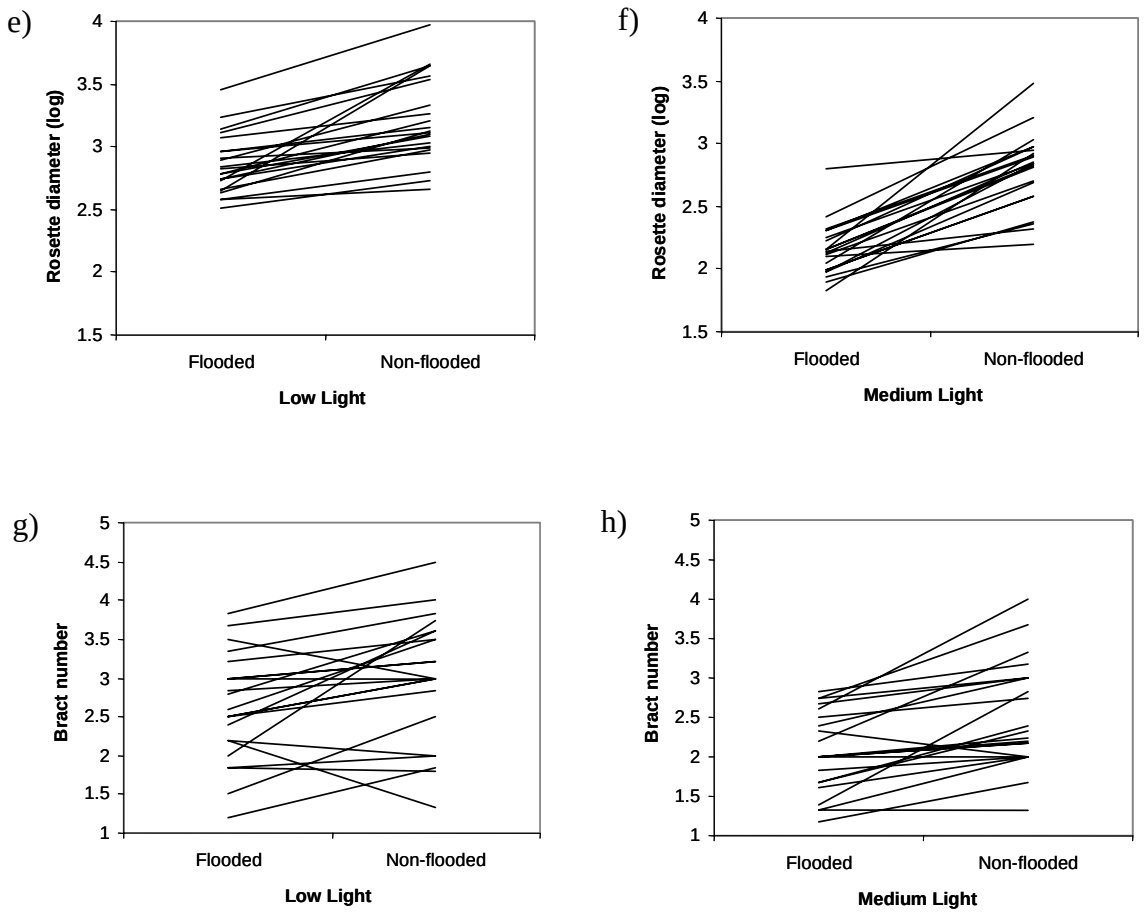

Figure 1. Continued.

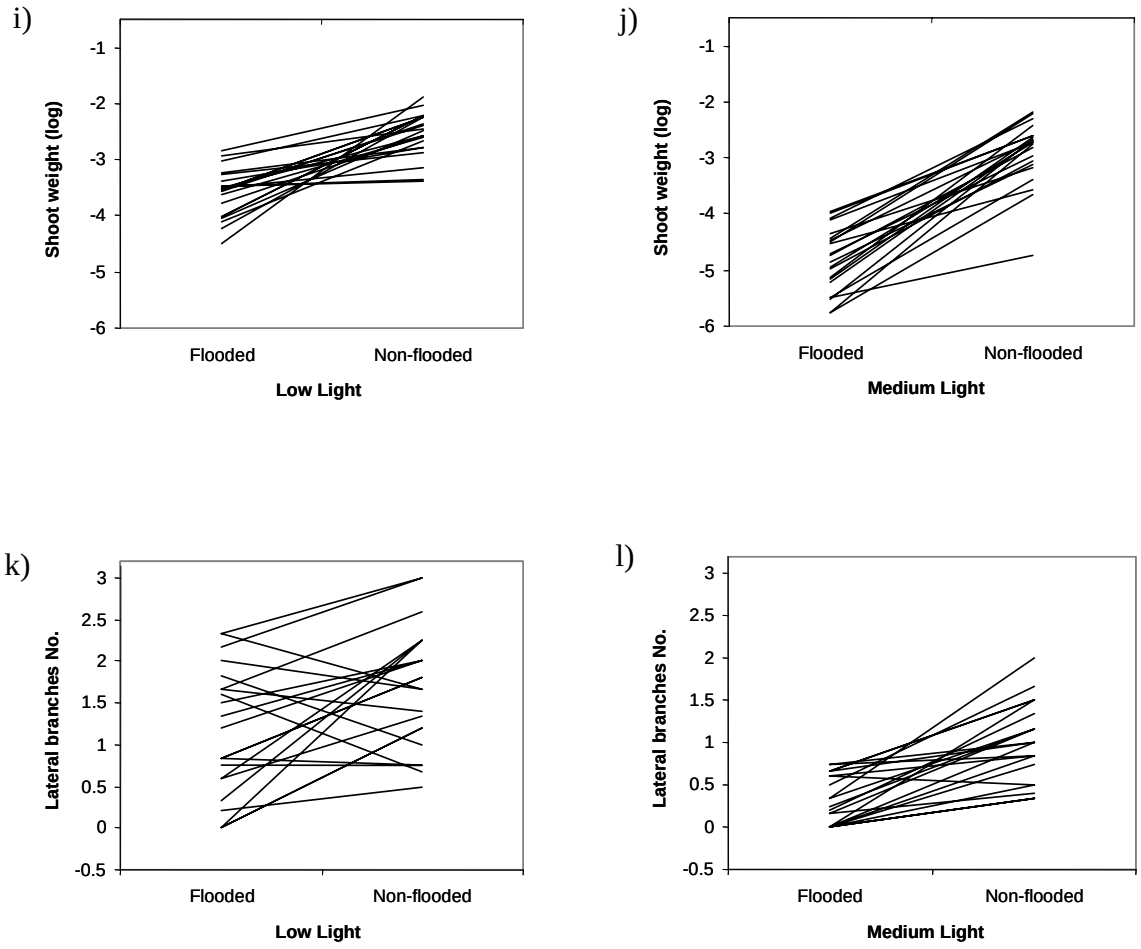

Figure 1. Continued.

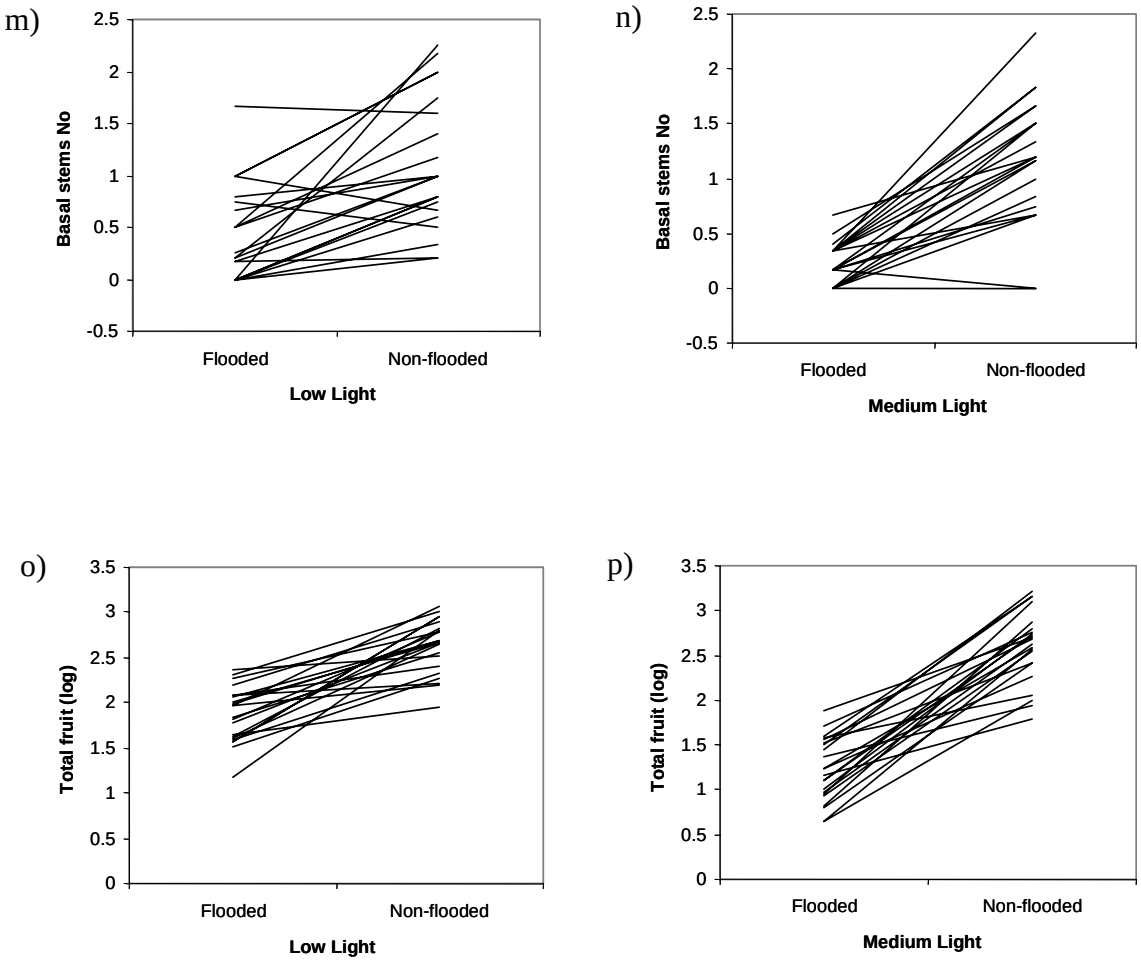

Figure 1. Continued.

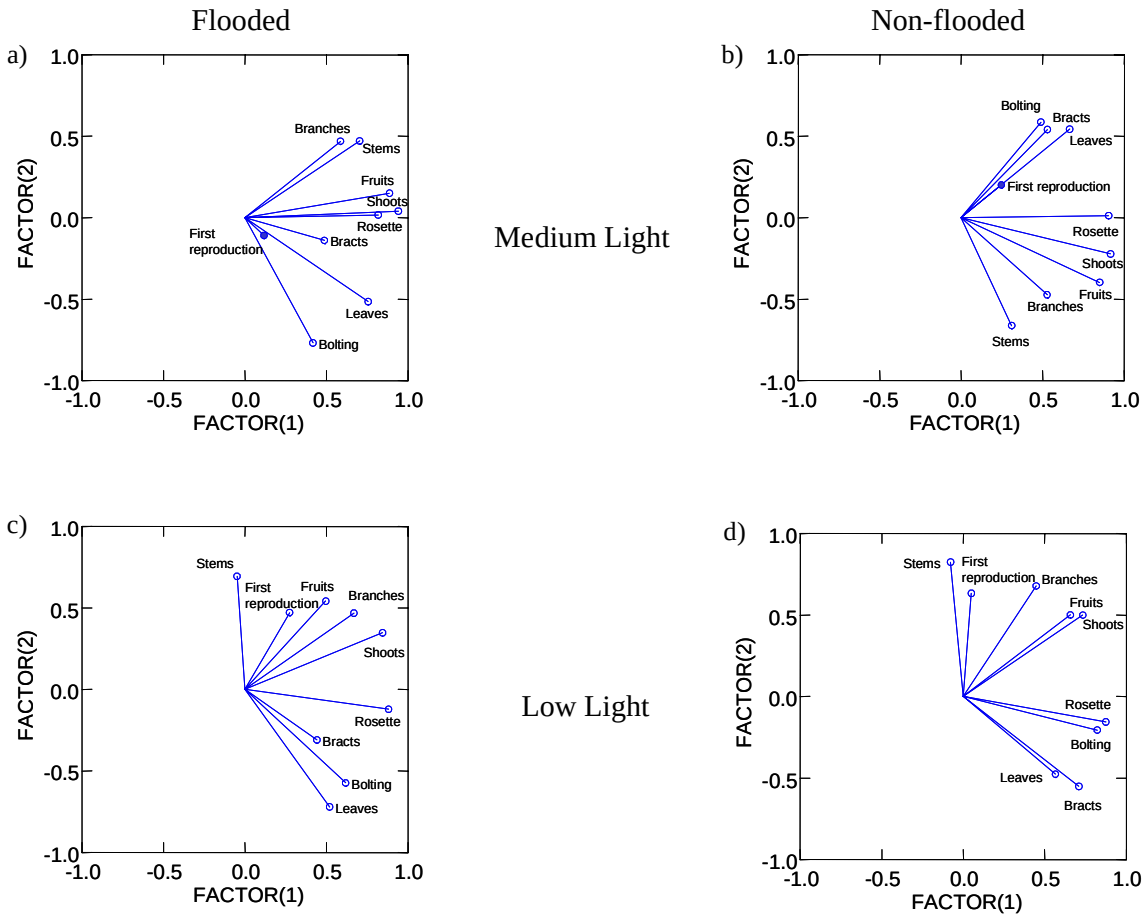

Figure 2. Principal components analyses of phenotypic integration as expressed under flooded (left) and non-flooded (right) conditions and under medium (top row) and low (bottom row) light intensity. The angles between vectors indicate the degree of independence of individual variables. Only the first two principal components are shown (see Tables 4-7 for the percentages of variance explained by each eigenvector).

VITA

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